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THE

BIOLOGICAL BULLETIN

PUBLISHED BY
THE MARINE BIOLOGICAL LABORATORY

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VOLUME LXVII

AUGUST TO DECEMBER, 1934

Printed and Issued by
LANCASTER PRESS, Inc.
PRINCE & LEMON STS.
LANCASTER, PA.

THE BIOLOGICAL BULLETIN is issued six times a year. Single numbers, $1.75. Subscription per volume (3 numbers), $4.50.

Subscriptions and other matter should be addressed to the Biological Bulletin, Prince and Lemon Streets, Lancaster, Pa. Agent for Great Britain: Wheldon & Wesley, Limited, 2, 3 and 4 Arthur Street, New Oxford Street, London, W.C. 2.

Communications relative to manuscripts should be sent to the Managing Editor, Marine Biological Laboratory, Woods Hole, Mass., between May 1 and October 1 and to the Institute of Biology, Divinity Avenue, Cambridge, Mass., during the remainder of the year.

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Entered October 10, 1902, at Lancaster, Pa., as second-class matter under Act of Congress of July 16, 1894.

LANCASTER PRESS, INC., LANCASTER, PA.

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THE
BIOLOGICAL BULLETIN

PUBLISHED BY THE MARINE BIOLOGICAL LABORATORY

BIOLOGICAL
LIBRARY

THE MARINE BIOLOGICAL LABORATORY

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FORTY-SIXTH YEAR

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II. ACT OF INCORPORATION

No. 3170

COMMONWEALTH OF MASSACHUSETTS

Be It Known, That whereas Alpheus Hyatt, William Sanford Stevens, William T. Sedgwick, Edward G. Gardiner, Susan Minns, Charles Sedgwick Minot, Samuel Wells, William G. Farlow, Anna D. Phillips and B. H. Van Vleck have associated themselves with the intention of forming a Corporation under the name of the Marine Biological Laboratory, for the purpose of establishing and maintaining a laboratory or station for scientific study and investigation, and a school for instruction in biology and natural history, and have complied with the provisions of the statutes of this Commonwealth in such case made and provided, as appears from the certificate of the President, Treasurer, and Trustees of said Corporation, duly approved by the Commissioner of Corporations, and recorded in this office;

Now, therefore, I, HENRY B. PIERCE, Secretary of the Commonwealth of Massachusetts, *do hereby certify* that said A. Hyatt, W. S. Stevens, W. T. Sedgwick, E. G. Gardiner, S. Minns, C. S. Minot, S. Wells, W. G. Farlow, A. D. Phillips, and B. H. Van Vleck, their associates and successors, are legally organized and established as, and are hereby made, an existing Corporation, under the name of the MARINE BIOLOGICAL LABORATORY, with the powers, rights, and privileges, and subject to the limitations, duties, and restrictions, which by law appertain thereto.

Witness my official signature hereunto subscribed, and the seal of the Commonwealth of Massachusetts hereunto affixed, this twentieth day of March, in the year of our Lord One Thousand Eight Hundred and Eighty-Eight.

[SEAL]

HENRY B. PIERCE,
Secretary of the Commonwealth.

III. BY-LAWS OF THE CORPORATION OF THE MARINE BIOLOGICAL LABORATORY

I. The annual meeting of the members shall be held on the second Tuesday in August, at the Laboratory, in Woods Hole, Mass., at 11.30 A.M., daylight saving time, in each year, and at such meeting the members shall choose by ballot a Treasurer and a Clerk to serve one year, and eight Trustees to serve four years. There shall be thirty-two Trustees thus chosen divided into four classes, each to serve four years, and in addition there shall be two groups of Trustees as follows: (*a*) Trustees *ex officio,* who shall be the

President of the Corporation, the Director of the Laboratory, the Associate Director, the Treasurer and the Clerk; (*b*) Trustees Emeritus, who shall be elected from the Trustees by the Corporation. Any regular Trustee who has attained the age of seventy years shall continue to serve as Trustee until the next annual meeting of the Corporation, whereupon his office as regular Trustee shall become vacant and be filled by election by the Corporation and he shall become eligible for election as Trustee Emeritus for life. The Trustees *ex officio* and Emeritus shall have all rights of the Trustees except that Trustees Emeritus shall not have the right to vote.

The Trustees and officers shall hold their respective offices until their successors are chosen and have qualified in their stead.

II. Special meetings of the members may be called by the Trustees to be held in Boston or in Woods Hole at such time and place as may be designated.

III. The Clerk shall give notice of meetings of the members by publication in some daily newspaper published in Boston at least fifteen days before such meeting, and in case of a special meeting the notice shall state the purpose for which it is called.

IV. Twenty-five members shall constitute a quorum at any meeting.

V. The Trustees shall have the control and management of the affairs of the Corporation; they shall present a report of its condition at every annual meeting; they shall elect one of their number President of the Corporation who shall also be Chairman of the Board of Trustees; they shall appoint a Director of the Laboratory; and they may choose such other officers and agents as they may think best; they may fix the compensation and define the duties of all the officers and agents; and may remove them, or any of them, except those chosen by the members, at any time; they may fill vacancies occurring in any manner in their own number or in any of the offices. They shall from time to time elect members to the Corporation upon such terms and conditions as they may think best.

VI. Meetings of the Trustees shall be called by the President, or by any two Trustees, and the Secretary shall give notice thereof by written or printed notice sent to each Trustee by mail, postpaid. Seven Trustees shall constitute a quorum for the transaction of business. The Board of Trustees shall have power to choose an Executive Committee from their own number, and to delegate to such Committee such of their own powers as they may deem expedient.

VII. The accounts of the Treasurer shall be audited annually by a certified public accountant.

VIII. The consent of every Trustee shall be necessary to dissolution of the Marine Biological Laboratory. In case of dissolution, the property shall be disposed of in such manner and upon such terms as shall be determined by the affirmative vote of two-thirds of the Board of Trustees.

IX. These By-laws may be altered at any meeting of the Trustees, provided that the notice of such meeting shall state that an alteration of the By-laws will be acted upon.

X. Any member in good standing may vote at any meeting, either in person or by proxy duly executed.

IV. THE REPORT OF THE TREASURER

To the Trustees of the Marine Biological Laboratory:

Gentlemen: Herewith is submitted my report as Treasurer of the Marine Biological Laboratory for the year 1933.

The accounts have been audited by Messrs. Seamans, Stetson, and Tuttle, certified public accountants, and a copy of their report is on file at the Laboratory and is open to inspection by members of the Corporation.

At the end of the year 1933 the book value of the Endowment Funds in the hands of the Central Hanover Bank and Trust Company was as follows:

General Fund,	securities	$ 912,230.91
	in cash	53.08
Library Fund,	securities	196,374.00
	in cash	70.00
Total book value		$1,108,727.99

The income collected from these Funds was as follows:

General Endowment	$40,528.28
Library	$ 8,538.89

Income in arrears, some of which may never be collected, was on December 31:

General Fund	$4,696.66
Library Fund	$1,253.50

The Reserve Fund was partially liquidated at a profit of over $800 and $11,000 applied to the reduction of the Devil's Lane mortgage, leaving a balance of securities and cash in the Fund at the end of the year of the book value of $11,921.17.

The Retirement Fund consisted of securities of the book value of	$20,060.00
Cash	3,103.66
Total	$23,163.66

Income in arrears on December 31 was $121.61.

The land, buildings, equipment and library, exclusive of the Gansett and Devil's Lane tracts, represented an investment of	$1,701,816.19
less reserve for depreciation	369,943.83
	$1,331,872.36

LIBRARY

Expenses, including $42,488.15 depreciation, exceeded income by $24,673.53. There was expended from current funds $18,798.63 in additions to Plant, mostly for books.

At the end of the year the Laboratory owed $14,000 on Bond and Mortgage, and $8,048.74 on open account and had $5,411.85 in cash and bank accounts.

Following is the balance sheet as of December 31, 1933, the condensed statement of income and outgo, also the surplus account, as set out by the accountants:

EXHIBIT A

MARINE BIOLOGICAL LABORATORY BALANCE SHEET, DECEMBER 31, 1933

Assets

Endowment Assets and Equities:			
Securities and Cash in Hands of Central Hanover Bank and Trust Company (New York), Trustee—Schedules I-a and I-b		$1,108,727.99	
Securities and Cash—Minor Funds—Schedule II		10,648.53	$1,119,376.52
Plant Assets:			
Land—Schedule IV	$ 98,103.05		
Buildings—Schedule IV	1,225,286.12		
Equipment—Schedule IV	170,329.50		
Library—Schedule IV	208,097.52	$1,701,816.19	
Less Reserve for Depreciation		369,943.83	
		$1,331,872.36	
Securities and Cash in Reserve Fund		11,921.17	
Cash in Dormitory Building Fund		713.96	$1,344,507.49
Current Assets:			
Cash		$ 5,411.85	
Accounts—Receivable		10,536.58	
Inventories:			
Supply Department	$ 38,513.31		
Biological Bulletin	9,755.26	48,268.57	
Investments:			
Devil's Lane Property	$ 42,345.83		
Gansett Property	5,250.49		
Stock in General Biological Supply House, Inc.	12,700.00		
Retirement Fund Assets	23,163.66	83,459.98	
Prepaid Insurance		3,400.46	
Items in Suspense (Net)		109.56	151,187.00

Liabilities

Endowment Funds:			
Endowment Funds—Schedule III		$1,108,727.99	
Minor Funds—Schedule III		10,648.53	$1,119,376.52
Plant Funds:			
Donations and Gifts—Schedule III		$1,029,372.61	
Other Investments in Plant from Gifts and Current Funds		314,134.88	
		$1,343,507.49	
Note—Payable, Danchakoff Estate		1,000.00	$1,344,507.49
Current Liabilities and Surplus:			
Mortgage, Devil's Lane Property		$ 14,000.00	
Accounts—Payable:			
Current	$ 3,376.24		
Hixon Electric Co., Deferred	3,672.50	7,048.74	
		21,048.74	
Current Surplus—Exhibit C		130,138.26	$ 151,187.00

EXHIBIT B

MARINE BIOLOGICAL LABORATORY INCOME AND EXPENSE, YEAR ENDED DECEMBER 31, 1933

	Total		Net	
	Expense	Income	Expense	Income
Income:				
General Endowment Fund		$ 40,528.28		$ 40,528.28
Library Fund		8,538.89		8,538.89
Gifts		400.00		400.00
Instruction	8,055.21	8,910.00		854.79
Research	4,017.04	12,535.50		8,518.46
Evening Lectures	99.41		99.41	
Biological Bulletin and Memship Dues	8,556.28	8,836.44		280.16
Supply Department—Schedule V	38,018.86	36,853.60	1,165.26	
Mess—Schedule VI	21,095.99	22,837.04		1,741.05
Dormitories—Schedule VII	31,093.23	12,531.56	18,561.67	
(Interest and Depreciation charged to above Three Departments. See Schedules V, VI, and VII)	*36.227.30*			36,227.30
Dividends, General Biological Supply House, Inc.		1,120.00		1,120.00
Rents:				
Danchakoff Cottages	294.31	637.50		343.19
Microscopes		284.20		284.20
Garage, Railway, etc.		142.00		142.00
Newman Cottage	124.04	250.00		125.96
Janitor's House	92.03	360.00		267.97

Interest on Bank Balances		29.96		29.96
Sales of Duplicate Library Sets		232.71		232.71
Sundries		26.16		26.16
Maintenance of Plant:				
New Laboratory Expenses	14,890.30		14,890.30	
Chemical and Special Apparatus	11,965.38		11,965.38	
Maintenance, Buildings and Grounds	6,764.24		6,764.24	
Library Department Expenses	7,505.98		7,505.98	
Carpenter Department Expenses	621.17		621.17	
Truck Expenses	914.11		914.11	
Sundry Expenses	83.63		83.63	
Workmen's Compensation Insurance	547.85		547.85	
Pumping Station Expenses	338.05		338.05	
General Expenses:				
Administration Expenses	14,454.27		14,454.27	
Endowment Fund Trustee	968.50		968.50	
Interest on Loans	75.80		75.80	
Bad Debts	620.67		620.67	
Reserve for Depreciation	42,488.15		42,488.15	
Museum Expenses	2,270.17		2,270.17	
	$179,727.37	$155,053.84	$124 334.61	$ 99,661.08
Excess of Expenses over Income carried to Current Surplus—Exhibit C		24,673.53		24,673.53
		$179,727.37		$124,334.61

EXHIBIT C

MARINE BIOLOGICAL LABORATORY, CURRENT SURPLUS ACCOUNT

YEAR ENDED DECEMBER 31, 1933

Balance, January 1, 1933		$122,306.03
Add:		
Reserve for Depreciation charged to Plant Funds		42,488.15
Cash Transferred from Reserve Fund and used to reduce Mortgage on Devil's Lane Land		11,000.00
		$175,794.18
Deduct:		
Payments from Current Funds during Year for Plant Assets as shown in Schedule IV:		
Buildings	$ 562.70	
Equipment	3,100.47	
Library Books, etc.	15,030.46	
	$18,693.63	

Payments on Danchakoff Mortgage from Current Cash		1,000.00	
Excess of Expenses over Income for Year as shown in Exhibit B		24,673.53	
Pensions and Allowances Paid	$2,210.02		
Less Income of Retirement Fund	921.26	1,288.76	45,655.92
Balance, December 31, 1933—Exhibit A			$130,138.26

Respectfully submitted,

LAWRASON RIGGS, JR.,

Treasurer.

V. REPORT OF THE LIBRARIAN

The Library budget until February 6, 1933, was in accordance with that of 1932, namely: total, $24,000; apportioned: current books, $300; serials, $6,000; current serial binding, $1,500; express, $300; supplies, $500; back sets, $6,850; salaries, $8,550. It became necessary in February, 1933, for the Laboratory to revise its budget with a view to greater economy, and $5,000 was thus deducted from the Library budget (February 6). This reduction sum was of necessity taken from the "back sets" apportionment, leaving the amount for the year $1,850. Orders already outstanding at this time were laid out on the budget as follows: current books, $218.14; serials, $5,440.71 (about); current binding, $1,404.02; express, $90.29; supplies, $215.70; back sets, $2,113.75 (about); salaries, $7,150; total, $16,632.61; credit from sale of duplicates, $232.71, or a total of $16,399.90. The budget, reduced in 1933 by one salary ($1,400, as explained in the Report for 1932) and again in February, 1933 ($5,000), to $17,600, gave at the time a balance of $1,200.10. Then in April, when very few of the bills had been received and paid, especially the German bills (the German publishers' time of billing has always been delayed until after the actual issue of their publications), the deflation of the American dollar began. How greatly the fall in the comparative value of American currency affected the Library budget is clearly shown by a comparison of the next figures, the money paid out, with the above figures as estimated: current books, $218.14; serials, $6,922.41; current binding, $1,404.02; express, $90.29; supplies, $215.70; back sets, $3,353.65; salaries, $7,150; total, $19,354.21. Adding to the total budget allowed, $17,600, the credit due to sales, this shows a total deficit of $1,521.50. The addition of the balance that we would have had, had the American dollar remained as it was at the beginning of the year, to the actual deficit as it occurred, gives the total loss to the Laboratory of $2,721.60 due to deflation.

Fortunately for us, the German scientific publications have been reduced in price twenty to thirty per cent over last year's price, and the amount per year is fixed. We hope also that the mark will not increase to a value greater than $.40 during the year. For these reasons, we expect to contine all our current subscriptions in 1934 as we did in 1933. The " back sets " budget must again be sacrificed in so far as is necessary to keep the current serials going. Also, the binding of many volumes of current serials (about 300) selected from the less used must be omitted for the present year.

The Library now contains 37,420 volumes (5,866 books and 31,554 serial volumes), and 81,208 reprints (2,532 bound). Eighty-six new books were purchased and 15 new serials were undertaken in January, 1933, so that the Library now subscribes to 346 current journals (15 " carry-on " book orders were completed and 1 serial dropped); receives by exchange 580 (13 new); and by gift, 211. Four back sets were completed and 8 back sets were partially completed.

For the Woods Hole Oceanographic Institution, there were purchased 18 new books and 3 back sets; 5 exchanges were undertaken. A complete rendering of the expenditure of the $1,000 allowed by the Woods Hole Oceanographic Institution for the year can also be made, although their fiscal year is not closed until February 28. The $1,000 was spent approximately as follows: books, $54.81; serials, $554.21; binding, $43.21; supplies, $3.00; back sets, $344.77. No separate account is to be reported of the total volumes so far purchased for the Woods Hole Oceanographic Institution, as the gain in number is recorded in the total accessions of the Library.

Several notable gifts were made during the year. By Professor Bradley M. Patten, many books and reprints from his father's library were presented. Professor H. McE. Knower gave about 25 books and several hundred reprints; Dr. Robert W. Hegner, several hundred reprints; Dr. E. W. Gudger, several hundred reprints; and Dr. Louis Murbach willed to us his books and pamphlets. From authors, there were received 22 new books, and from publishers as follows: P. Blakiston's Son & Co., 1; Harper & Bros., 4; Lea & Febiger, 3; McGraw-Hill Book Co., Inc., 2; Macmillan Co., 2.

VI. THE REPORT OF THE DIRECTOR

To the Trustees of the Marine Biological Laboratory:

Gentlemen: I beg to submit herewith a report of the forty-sixth session of the Marine Biological Laboratory for the year 1933.

1. *Attendance.* The number of investigators in attendance in 1933 was slightly greater than that in 1932, the figures for the two years being 319 and 314, respectively. At the same time, the registration in the courses of instruction fell from 132 to 118, giving after a correction for duplications a net decrease of 7 in the total attendance. The number of institutions represented by students in the courses was 58 in 1933 as compared with 76 in 1932, but that represented by investigators remained almost unchanged, being 2 less than that of the preceding year and only 10 less than the maximum of 102 reached in 1931. The seasonal distribution of the attendance in 1933, and for comparison, that for the preceding six years, is shown in tabular form.

		1927	1928	1929	1930	1931	1932	1933
May	30	7	15	9	6	6	8	11
June	10	50	64	55	50	51	54	46
"	20	114	140	139	153	153	127	129
"	30	212	240	197	208	217	172	184
July	10	247	281	238	253	258	225	235
"	20	247	282	242	250	273	245	253
"	30	245	272	249	253	281	248	255
August	10	234	250	256	254	302	257	261
"	20	208	226	243	245	280	236	244
"	30	168	183	220	204	239	190	205
September	10	110	112	157	122	136	129	117
"	20	50	43	59	44	69	58	45
"	30	12	14	14	8	14	13	12

2. *The Report of the Treasurer.* As in the case of nearly all educational and scientific institutions, the year 1933 was a particularly unfavorable one for the Laboratory from a financial, though fortunately not from a scientific point of view. The total gross income, which in 1932 had shown a decrease of approximately $29,000.00 from the previous year, declined still further by about $22,000.00 to $155,053.84, which is the lowest figure reached since the establishment of the present endowment in 1925. The loss in gross income was accounted for roughly as follows: income from investments, $6,600.00; subscriptions from institutions, $900.00; sales by the Supply Department, $7,100.00; receipts from the Mess and the dormitories, $4,400.00; miscellaneous, $3,000.00. As to the first of these items, it should be noted that a decrease in income from investments of only approximately 12 per cent

under the conditions prevailing in 1933 indicates an exceptional soundness of the securities in the endowment fund. Few endowed institutions have fared so well. It should be noted further that at least a part of the usual income from the endowment fund which was not received in 1933 will eventually be paid, though to balance this favorable circumstance is the unfavorable one that funds now invested in securities about to mature cannot with equal safety be reinvested so as to yield their present rates of interest. The attention of all the members of the Corporation is invited to the possible opportunities they may have, through the institutions with which they are connected, to prevent further losses in income from the second and third of the sources mentioned above.

In order to carry on its activities within its considerably reduced budget, the Laboratory in 1933 adopted certain economies which, though unwelcome, did not seriously handicap its scientific work. The most important of these was the discontinuance of the use of the steamer "Cayadetta," which to many persons had seemed to be an indispensable part of the equipment of the institution. The experience of the past year, however, has shown that at least the most important scientific needs of the investigators and of the students in the courses can be cared for much less expensively than previously by means of smaller boats, including both those already belonging to the Laboratory and others rented from time to time for special occasions. By means of this major and many minor economies, the expenditures in 1933 were reduced to the point where, except for depreciation charges, the budget was balanced. As has been mentioned in previous reports, the depreciation charges on the expensive buildings and equipment owned by the Laboratory cannot, under present conditions, be met from current income, and an annual deficit in a strict business sense is therefore unavoidable. This deficit for 1933 amounted to $24,673.53.

One of the most favorable points mentioned in the Treasurer's Report is the payment by the Laboratory during 1933 of a considerable part of its indebtedness on the Devil's Lane Tract. Further payments made after the period covered by the report on this and on one other outstanding mortgage have reduced the total amount owed by the Laboratory on mortgages from $27,000.00 in the summer of 1933 to only $2,000.00 in May, 1934. The future savings resulting from the almost complete elimination of interest payments at rates higher than those at present obtainable from first-class securities will be most advantageous to the Laboratory.

3. *The Report of the Librarian.* The Library, handicapped by a reduction of $5,000.00 in its appropriation for the purchase of back sets of journals and by the low purchasing power of the dollar in foreign

countries, has nevertheless maintained, though at a somewhat slower rate, the steady progress it has shown for a number of years past. Its growth since 1925 can most concisely be shown by means of the following table:

	1925	1926	1927	1928	1929	1930	1931	1932	1933
Serials received currently	500	628	764	874	985	1,060	1,080	1,126	1,137
Total number of bound volumes	15,000	18,200	22,800	26,500	28,300	31,500	33,800	36,000	37,400
Reprints . .	25,000	38,000	43,000	51,000	59,000	64,000	70,000	76,000	81,000

Appreciative acknowledgment may be made at this point of the generosity of those mentioned in the Librarian's Report who, during the past year, have presented valuable books and reprints to the Library.

4. *Lectures and Scientific Meetings.* During the summer of 1933, 10 regular evening lectures and also several special lectures were given, and 12 evening scientific sessions were held at which 48 shorter papers on recently completed work were presented by investigators associated with the Marine Biological Laboratory and the Woods Hole Oceanographic Institution. As for several years past, an important feature of the scientific program of the summer was an all-day session held on August 31 at which 31 additional papers, based on work done at the Laboratory during the current season, were presented. Abstracts of most of the shorter scientific contributions will be found in the Biological Bulletin for October, 1933.

5. *Retirement of Employees.* During the past year three persons who have been associated with the Laboratory almost from its beginning retired from active service. Few institutions have been so fortunate in the length and the loyalty of the services of their employees as has the Marine Biological Laboratory in the cases of John J. Veeder, George M. Gray, and Ellis M. Lewis. In recognition of this fact, suitably engrossed letters of appreciation were presented by the Board of Trustees to those retiring, and these letters are here placed on permanent record:

On the Occasion of the Retirement of

CAPTAIN JOHN VEEDER

from active service of the

MARINE BIOLOGICAL LABORATORY

the Trustees offer him their thanks for the faithful performance of his responsible duties as Captain of the Laboratory Boats for the past

Forty-five Years.

They congratulate him upon his PERFECT RECORD of never having had a serious accident either to life or property, although during these many years he has carried thousands of students and investigators on Collecting Trips and Excursions.

The many friends of "Captain John" unite in wishing for him Health, Happiness and the satisfaction that comes from Work Well Done.

The Trustees of the
Marine Biological Laboratory
hereby express to

GEORGE MILTON GRAY

their satisfaction and pride in his faithful and efficient services, extending over a period of

Forty-two Years

first as Collector, then as Manager of the Supply Department, and finally as Curator of the Museum.

Under his management the Supply Department became an important part of the work of the Laboratory and by his kindness and courtesy Mr. Gray has endeared himself to a host of students and investigators.

On the occasion of his retirement from active service, the Trustees extend to him and to Mrs. Gray their best wishes for continued health and happiness.

"Well done, Good and Faithful Servant."

The Trustees of the
Marine Biological Laboratory
hereby record their appreciation
of the faithful services of

ELLIS M. LEWIS

who for a period of

Thirty-six Years

served the Laboratory as

CHIEF ENGINEER OF THE LABORATORY BOATS.

On the Occasion of his Retirement from Active Duties they extend to him their best wishes for continued usefulness and happiness.

6. *The Board of Trustees.* At the Annual Meeting of the Corporation held on Tuesday, August 8, Dr. William Ruthrauff Amberson of the University of Tennessee and Dr. Carl Caskey Speidel of the University of Virginia were elected Trustees to fill vacancies in the Class of 1937.

There are appended as parts of this Report:

1. The Staff, 1933.
2. Investigators and Students, 1933.
3. A Tabular View of Attendance, 1929–1933.
4. Subscribing and Coöperating Institutions, 1933.
5. Evening Lectures, 1933.
6. Shorter Scientific Papers, 1933.
7. Members of the Corporation, August, 1933.

Respectfully submitted,

M. H. JACOBS,

Director.

MARINE BIOLOGICAL LABORATORY LIBRARY WOODS HOLE, MASS.

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1. THE STAFF, 1933

Merkel H. Jacobs, *Director,* Professor of General Physiology, University of Pennsylvania.

Associate Director: ——

ZOÖLOGY

I. Investigation

Gary N. Calkins, Professor of Protozoölogy, Columbia University.
E. G. Conklin, Professor of Zoölogy, Princeton University.
Caswell Grave, Professor of Zoölogy, Washington University.
H. S. Jennings, Professor of Zoölogy, Johns Hopkins University.
Frank R. Lillie, Professor of Embryology, The University of Chicago.
C. E. McClung, Professor of Zoölogy, University of Pennsylvania.
S. O. Mast, Professor of Zoölogy, Johns Hopkins University.
T. H. Morgan, Director of the Biological Laboratory, California Institute of Technology.
G. H. Parker, Professor of Zoölogy, Harvard University.
E. B. Wilson, Professor of Zoölogy, Columbia University.
Lorande L. Woodruff, Professor of Protozoölogy, Yale University.

II. Instruction

T. H. Bissonnette, Professor of Biology, Trinity College.
E. C. Cole, Professor of Biology, Williams College.
B. R. Coonfield, Instructor in Biology, Brooklyn College.
C. E. Hadley, Associate Professor of Biology, New Jersey State Teachers College at Montclair.

O. E. Nelsen, Instructor in Zoölogy, University of Pennsylvania.
S. A. Matthews, Associate in Anatomy, School of Medicine, University of Pennsylvania.
L. P. Sayles, Instructor in Biology, College of the City of New York.

Junior Instructors

F. R. Hayes, Associate Professor of Zoölogy, Dalhousie University.
F. H. Woods, Assistant Professor of Zoölogy, University of Missouri.

PROTOZOÖLOGY

I. Investigation

(*See Zoölogy*)

II. Instruction

Gary N. Calkins, Professor of Protozoölogy, Columbia University.
Rachel Bowling, Instructor in Zoölogy, Columbia University.
Robert W. Stabler, Instructor in Zoölogy, University of Pennsylvania.

EMBRYOLOGY

I. Investigation

(*See Zoölogy*)

II. Instruction

L. G. Barth, Instructor of Experimental Zoölogy, Columbia University.
Hubert B. Goodrich, Professor of Biology, Wesleyan University.
Benjamin H. Grave, Professor of Biology, De Pauw University.
Leigh Hoadley, Professor of Zoölogy, Harvard University.
Charles Packard, Assistant Professor of Zoölogy, Institute of Cancer Research, Columbia University.

PHYSIOLOGY

I. Investigation

Harold C. Bradley, Professor of Physiological Chemistry, University of Wisconsin.
Walter E. Garrey, Professor of Physiology, Vanderbilt University Medical School.
Ralph S. Lillie, Professor of General Physiology, The University of Chicago.
Albert P. Mathews, Professor of Biochemistry, University of Cincinnati.

II. Instruction

Teaching Staff

William R. Amberson, Professor of Physiology, University of Tennessee.
Robert Chambers, Professor of Biology, New York University.
Ralph W. Gerard, Associate Professor of Physiology, The University of Chicago.

Laurence Irving, Associate Professor of Physiology, University of Toronto.
Balduin Lucké, Associate Professor of Pathology, University of Pennsylvania.
Leonor Michaelis, Member of the Rockefeller Institute, New York City.
Margaret Sumwalt, Assistant Instructor, University of Pennsylvania Medical School.

BOTANY

I. Investigation

C. E. Allen, Professor of Botany, University of Wisconsin.
S. C. Brooks, Professor of Zoölogy, University of California.
B. M. Duggar, Professor of Physiological and Economic Botany, University of Wisconsin.
Ivey F. Lewis, Professor of Biology, University of Virginia.
Wm. J. Robbins, Professor of Botany, University of Missouri.

II. Instruction

William Randolph Taylor, Professor of Botany, University of Michigan.
Hugh P. Bell, Professor of Botany, Dalhousie University.
G. W. Prescott, Assistant Professor of Biology, Albion College.

GENERAL OFFICE

F. M. MacNaught, Business Manager.
Polly L. Crowell, Assistant.

RESEARCH SERVICE AND GENERAL MAINTENANCE

Samuel E. Pond, Technical Manager.
Oscar W. Richards, Chemical Service.
G. Failla, X-Ray Physicist.
Thomas E. Larkin, Superintendent.
William Hemenway, Carpenter.
Lester F. Boss, Research Technician.
J. D. Graham, Glassblower.
P. H. Liljestrand, Assistant.

LIBRARY

Priscilla B. Montgomery (Mrs. Thomas H. Montgomery, Jr.), Librarian.
Deborah Lawrence, Secretary.
Doris Endrejat, Mary A. Rohan, Assistants.

SUPPLY DEPARTMENT

James McInnis, Manager.
John J. Veeder, Captain.
Ellis M. Lewis, Engineer.
A. M. Hilton, Collector.
A. W. Leathers, Shipping Department.
Milton B. Gray, Collector.
Geoffrey Lehy, Collector.
Walter Kahler, Collector.

MUSEUM

George M. Gray, Curator.

2. INVESTIGATORS AND STUDENTS, 1933

Independent Investigators

Adams, Edgar M., Graduate Assistant, University of Cincinnati.
Adams, Theodore G., Instructor in Biology, John Adams High School.
Addison, William H. F., Professor of Normal Histology and Embryology, University of Pennsylvania.
Amberson, William R., Professor of Physiology, University of Tennessee.
Anderson, Rubert S., Research Associate, Princeton University.
Armstrong, Philip B., Assistant Professor of Anatomy, Cornell University Medical College.
Bacq, Z. M., Assistant in Laboratory of Physiology, University of Liège, Liège, Belgium.
Bailey, Ralph J., Instructor in Zoölogy. George Washington University.
Baitsell, George A., Professor of Biology, Yale University.
Ball, Eric G., Instructor in Chemistry, Johns Hopkins Medical School.
Barron, E. S. Guzman, Assistant Professor of Biochemistry, The University of Chicago.
Barth, L. G., Instructor, Columbia University.
Beams, H. W., Assistant Professor of Zoölogy, State University of Iowa.
Bell, Hugh P., Professor of Botany, Dalhousie University.
Bissonnette, T. Hume, Professor of Biology, Trinity College.
Blinks, Lawrence R., Associate, General Physiology, Rockefeller Institute.
Bodansky, Aaron, Biochemist, Research Laboratory, Hospital for Joint Diseases, New York City, N. Y.
Bodine, J. H., Professor and Head of Zoölogy, State University of Iowa.
Bowen, Rufus E., Assistant Professor of Biology, Long Island University.
Bowling, Rachel, Instructor in Zoölogy, Columbia University.
Bradley, H. C., Professor of Physiological Chemistry, University of Wisconsin.
Brinley, F. J., Assistant Professor of Zoölogy and Physiology, North Dakota State College.
Brooks, M. M., Research Associate in Biology, University of California.
Brooks, S. C., Professor of Zoölogy, University of California.
Brown, Dugold E. S., Assistant Professor of Physiology, New York University and Bellevue Medical College.
Budington, R. A., Professor of Zoölogy, Oberlin College.
Bulmer, Gladys, Teacher of Biology, University of Pennsylvania.
Butler, Elmer G., Associate Professor of Biology, Princeton University.
Calkins, Gary N., Professor of Protozoölogy, Columbia University.
Cannan, Robert K., Professor of Chemistry, University and Bellevue Hospital Medical College.
Carpenter, Russell L., Associate in Anatomy, College of Physicians and Surgeons, Columbia University.
Cattell, Ware, Garrison-on-Hudson, New York.
Chambers, Robert, Research Professor of Biology, Washington Square College, New York University.
Cheney, Ralph H., Chairman, Biology Department, Long Island University.
Chesley, Leon C., Assistant Biophysicist, Memorial Hospital.
Chidester, F. E., Professor of Zoölogy, West Virginia University.
Clark, Eleanor L., University of Pennsylvania.
Clark, Eliot R., Professor of Anatomy, University of Pennsylvania.
Clowes, George H. A., Director of Research, Lilly Research Laboratories.
Coe, W. R., Professor of Biology, Yale University.
Coghill, George E., Member, Wistar Institute of Anatomy and Biology.
Cole, Elbert C., Professor of Biology, Williams College.

Collip, J. B., Professor of Biochemistry, McGill University.
Conklin, E. G., Professor of Biology, Princeton University.
Coonfield, Benjamin R., Instructor in Biology, Brooklyn College.
Copeland, Manton, Professor of Biology, Bowdoin College.
Cowles, Rheinart P., Professor of Zoölogy, Johns Hopkins University.
Croasdale, Hannah T., Graduate Student, University of Pennsylvania.
Dawson, Alden B., Associate Professor of Zoölogy, Harvard University.
Day, Dorothy, Assistant Professor of Botany, Smith College.
Diller, William F., Instructor in Zoölogy, Dartmouth College.
Donaldson, Henry H., Member, Wistar Institute of Anatomy and Biology.
Donaldson, John C., Professor of Anatomy, University of Pittsburgh, Medical School.
Edwards, Dayton J., Associate Professor of Physiology, Cornell University Medical College.
Edwards, Thomas I., Instructor in Biology, School of Hygiene, Johns Hopkins University.
Enders, Robert K., Assistant Professor of Zoölogy, Swarthmore College.
Finley, Harold E., Associate Professor of Biology, West Virginia State College.
Fleisher, Moyer S., Professor of Bacteriology and Hygiene, St. Louis University.
Fowler, Virginia M., Assistant in Botany, Barnard College.
Fry, Henry J., Professor of Biology, Washington Square College, New York University.
Furth, Jacob, Assistant Professor in Pathology, Cornell University Medical College.
Furtos, Norma C., Fellow in Biology, Western Reserve University.
Garrey, W. E., Professor of Physiology, Vanderbilt University, School of Medicine.
Gates, Gordon E., Head of Department of Biology, Judson College.
Geiman, Quentin M., Research Assistant, University of Pennsylvania.
Gerard, R. W., Associate Professor of Physiology, The University of Chicago.
Gilmore, Kathryn A., Instructor in Botany, Pennsylvania College for Women.
Goldforb, A. J., Professor of Biology, College of the City of New York.
Goodrich, H. B., Professor of Biology, Wesleyan University.
Graubard, Marc A., Department of Zoölogy, Columbia University.
Grave, B. H., Professor of Zoölogy, DePauw University.
Grave, Caswell, Professor of Zoölogy, Washington University.
Hadley, Charles E., Associate Professor of Biology, State Teachers College at Montclair.
Hall, Edmund K., Instructor, University of Louisville, School of Medicine.
Hamburger, Victor, Assistant, University of Frieburg, Frieburg, Germany.
Harnly, Morris H., Assistant Professor, Washington Square College, New York University.
Hartline, H. K., Lecturer in Medical Physics, University of Pennsylvania.
Harvey, Ethel Browne, Research Worker, Princeton University.
Harvey, E. Newton, Professor of Physiology, Princeton University.
Hayes, Frederick R., Associate Professor of Zoölogy, Dalhousie University.
Haywood, Charlotte, Associate Professor of Physiology, Mount Holyoke College.
Heilbrunn, L. V., Associate Professor of Zoölogy, University of Pennsylvania.
Henshaw, P. S., Biophysicist, Memorial Hospital, New York City.
Hetherington, W. Alford, Bruce Fellow, Johns Hopkins University.
Hibbard, Hope, Associate Professor, Oberlin College.
Hill, Samuel E., Assistant in General Physiology, Rockefeller Institute.
Hoadley, Leigh, Professor of Zoölogy, Harvard University.
Hollaender, Alexander, National Research Fellow in Biological Science, University of Wisconsin.

Hoppe, Ella N., Bacteriologist, New York State Department of Health.
Howard, Evelyn, Instructor in Physiology, Johns Hopkins Medical School.
Howe, H. E., Editor, Industrial and Engineering Chemistry.
Hussey, Kathleen L., Assistant in Zoölogy, Connecticut College.
Hutner, Seymour H., Graduate Student, Cornell University.
Hyde, Ida H., Emeritus Professor of Physiology, State University of Kansas.
Irving, Laurence, Associate Professor of Physiology, University of Toronto.
Jacobs, M. H., Professor of General Physiology, University of Pennsylvania.
Jasper, Herbert H., Research Fellow, National Research Council and Brown University.
Jenkins, George B., Professor and Director Department of Anatomy, George Washington University.
Jennings, H. S., Professor of Zoölogy, Johns Hopkins University.
Johlin, J. M., Associate Professor of Biochemistry, Vanderbilt Medical School.
Keil, Elsa Marie, Instructor in Zoölogy, New Jersey College for Women.
Kelley, Truman L., Professor in Graduate School of Education, Harvard University.
Keyes, Donald B., Professor of Chemical Engineering, University of Illinois.
Kidder, George W., Tutor, College of the City of New York.
Kirkpatrick, T. Bruce, Associate Professor of Physical Education, Columbia University.
Knowlton, Frank P., Professor of Physiology, Syracuse University, College of Medicine.
Korr, Irvin M., Assistant Instructor, Princeton University.
Krogh, Marie, University of Copenhagen, Denmark.
Lancefield, D. E., Associate Professor of Zoölogy, Columbia University.
Lancefield, Rebecca C., Associate in Bacteriology, Rockefeller Institute for Medical Research.
Laug, Edwin P., Instructor in Physiology, University of Pennsylvania.
Lillie, Frank R., Chairman of the Department of Zoölogy, The University of Chicago.
Lillie, Margaret Crane, Boston City Hospital.
Lillie, Ralph S., Professor of General Physiology, The University of Chicago.
Lucké, Balduin, Professor of Pathology, University of Pennsylvania.
Lynch, Ruth Stocking, Instructor, Johns Hopkins University.
MacDougall, Mary Stuart, Professor of Zoölogy, Agnes Scott College.
McGregor, J. H., Professor of Zoölogy, Columbia University.
Machlis, Samuel, Graduate Assistant in Biology, Washington Square College, New York University.
Magruder, Samuel R., Graduate Assistant in Zoölogy, University of Cincinnati.
Martin, Earl A., Assistant Professor, Chairman of Biology Department, Brooklyn College.
Mast, S. O., Professor of Zoölogy, Johns Hopkins University.
Mathews, A. P., Carnegie Professor of Biochemistry, University of Cincinnati.
Matthews, Samuel A., Associate in Anatomy, University of Pennsylvania.
Menkin, Valy, Instructor in Pathology, Harvard University Medical School.
Michaelis, Dr. Leonor, Member, Rockefeller Institute for Medical Research.
Miller, Forrest W., Graduate Assistant, University of Pittsburgh.
Moment, Gairdner B., Instructor in Biology, Goucher College.
Moreland, Ferrin B., Assistant in Biochemistry, Vanderbilt Medical School.
Morgan, Lilian V., California Institute of Technology.
Morgan, T. H., Professor of Biology, California Institute of Technology.
Morgulis, Sergius, Professor of Biochemistry, University of Nebraska, College of Medicine.
Morrill, E. V., Associate Professor of Anatomy, Cornell University Medical College.

Nelsen, Olin E., Instructor in Zoölogy, University of Pennsylvania.
Nonidez, José F., Assistant Professor of Anatomy, Cornell University Medical College.
Northrop, John H., Member, Rockefeller Institute.
Oltmann, Clara E., Tutor, Brooklyn College.
Orr, Paul R., Instructor, Brooklyn College.
Packard, Charles, Assistant Professor of Zoölogy, Columbia University.
Palmer, A. H., Teaching Fellow, New York University and Bellevue Medical College.
Palmer, Elizabeth T., Instructor, Vassar College.
Papenfuss, George F., Instructor in Botany, Johns Hopkins University.
Parker, G. H., Professor of Zoölogy, Harvard University.
Parpart, Arthur K., Assistant Professor of Physiology, Princeton University.
Pelluet, Dixie, Assistant Professor of Zoölogy, Dalhousie University.
Plough, Harold H., Professor of Biology, Amherst College.
Pollister, Arthur W., Instructor in Zoölogy, Columbia University.
Pollister, Priscilla Frew, Instructor in Biology, Brooklyn College.
Pond, Samuel E., Technical Manager, Marine Biological Laboratory.
Prescott, G. W., Assistant Professor of Biology, Albion College.
de Renyi, George S., Associate Professor of Anatomy, University of Pennsylvania.
Rex, Richard O., Instructor in Anatomy, University of Pennsylvania.
Reznikoff, Paul, Assistant Professor of Medicine, Cornell University Medical College.
Richards, Oscar W., Instructor in Biology, Yale University.
Root, Walter S., Associate Professor of Physiology, College of Medicine, Syracuse University.
Rugh, Roberts, Instructor in Zoölogy, Hunter College.
Sayles, Leonard P., Instructor in Biology, College of the City of New York.
Schechter, Victor, Columbia University.
Schmidt, Ida T. Genther, Instructor in Histology, Children's Hospital, Cincinnati.
Schmidt, L. H., Fellow, Christ Hospital, Cincinnati.
Schmieder, Rudolf G., Instructor in Zoölogy, University of Pennsylvania.
Schotte, Oscar E., Sterling Research Fellow, Yale University.
Schrader, Franz, Professor of Zoölogy, Columbia University.
Schrader, Sally Hughes, Professor of Zoölogy, Sarah Lawrence College.
Scott, Allan C., Assistant in Zoölogy, Columbia University.
Scott, Sister Florence Marie, Graduate Student, Columbia University.
Shoup, Charles S., Assistant Professor of Biology, Vanderbilt University.
Shumway, Waldo, Professor of Zoölogy, University of Illinois.
Sichel, Ferdinand J. M., Assistant Instructor, Washington Square College, New York University.
Slifer, Eleanor H., Research Associate in Zoölogy, State University of Iowa.
Smith, Dietrich C., Harvard University.
Smith, Philip E., Professor of Anatomy, Columbia University.
Snell, George D., National Research Fellow, University of Texas.
Sonneborn, Tracy M., Research Associate, Johns Hopkins University.
Speicher, B. R., Graduate Assistant, University of Pittsburgh.
Speidel, Carl C., Professor of Anatomy, University of Virginia Medical School.
Spek, Josef, Professor of Zoölogy, University of Heidelberg, Germany.
Stabler, Robert M., Instructor in Zoölogy, University of Pennsylvania.
Steinbach, H. Burr, National Research Fellow, The University of Chicago.
Stewart, Dorothy R., Assistant Professor of Biology, Skidmore College.
Stockard, Charles R., Professor of Anatomy, Cornell University Medical College.
Stokey, Alma G., Professor of Botany, Mount Holyoke College.

Stunkard, Horace W., Professor of Biology, New York University.
Sturtevant, A. H., Professor of Genetics, California Institute of Technology.
Sumwalt, Margaret, Assistant Instructor, University of Pennsylvania Medical School.
Sweadner, Walter R., Graduate Assistant, University of Pittsburgh.
Tashiro, Dr. Shiro, Professor of Biochemistry, University of Cincinnati, College of Medicine.
Taylor, G. Wellford, National Research Fellow, Princeton University.
Taylor, William R., Professor of Botany, University of Michigan.
Tennent, David H., Professor of Biology, Bryn Mawr College.
Tulloch, George S., Instructor, Brooklyn College.
Van Cleave, Harley J., Professor of Zoölogy, University of Illinois.
Vicari, Emilia M., Associate in Anatomy, Cornell University Medical College.
Waterman, Allyn J., Instructor in Biology, Brooklyn College.
Weichert, Charles K., Assistant Professor of Zoölogy, University of Cincinnati.
Weisman, Maxwell N., Tutor, College of the City of New York.
Whedon, Arthur D., Professor and Head of Zoölogy and Physiology, North Dakota Agricultural College.
Whiting, Anna R., Professor and Head of Department of Biology, Pennsylvania College for Women.
Whiting, P. W., Professor of Zoölogy, University of Pittsburgh.
Wieman, H. L., Professor of Zoölogy, University of Cincinnati.
Willey, Charles H., Assistant Professor of Biology, University College, New York University.
Wilson, E. B., Da Costa Professor Emeritus in Residence, Columbia University.
Winsor, Charles P., Student, Harvard University.
Wolf, E. Alfred, Associate Professor of Zoölogy, University of Pittsburgh.
Woods, Farris H., Assistant Professor of Zoölogy, University of Missouri.
Woodward, Alvalyn E., Assistant Professor, University of Michigan.
Young, Roger Arliner, Assistant Professor of Zoölogy, Howard University.
Young, William C., Assistant Professor of Biology, Brown University.
Zirkle, Conway, Associate Professor, University of Pennsylvania.
Zirkle, Raymond E., National Research Council Fellow, Johnson Foundation, University of Pennsylvania.

Beginning Investigators

Abramowitz, Alexander A., Harvard University.
Agate, Frederic J., Graduate Student, Columbia University.
Albaum, Harry G., Fellow, Brooklyn College.
Atlas, Meyer, Assistant in Zoölogy, Columbia University.
Baden, Victor, Graduate Student and University Scholar, University of Pennsylvania.
Beck, Lyle V., Graduate Assistant in Zoölogy, University of Pittsburgh.
Bostian, Carey H., Assistant Professor of Zoölogy, North Carolina State College.
Bridges, John C., Instructor in Zoölogy, Morehouse College.
Carlson, J. Gordon, Instructor in Biology, Bryn Mawr College.
Carleton, Blondel H., Fellow in Physiology, University of Rochester Medical School.
Chao, Iping, Graduate Student, The University of Chicago.
Chen, Tze-Tuan, Instructor, University of Pennsylvania.
Clark, Jean M., Graduate Student, University of Pennsylvania.
Cohen, Rose S., Graduate Assistant in Zoölogy, University of Cincinnati.
Cole, Margaret Grierson, Mount Holyoke College.
Costello, Donald P., Instructor in Zoölogy, University of Pennsylvania.
Crampton, C. B., Instructor in Biology, Wesleyan University.
Dan, Katsuma, Graduate Student, University of Pennsylvania.

Derrickson, Mary B., Frederica, Delaware.
Drumtra, Elizabeth, Assistant in Zoölogy, Barnard College, Columbia University.
Duncan, Paul M., Graduate Student, University of Pennsylvania.
Eastlick, Herbert L., Graduate Assistant in Zoölogy, Washington University.
Farrow, John G., High School Instructor and Student, University of Pennsylvania.
Havey, Clinton B., Acadia University.
Hegnauer, Albert H., Assistant in Physiology, University of Rochester.
Hicks, Frederick James, Graduate Student in Zoölogy, University of Pittsburgh.
Hirschfeld, Nathan B., Graduate Student, Columbia University.
Hunter, Laura, Graduate Student, University of Pennsylvania.
Johnson, Arlene C., Graduate Assistant, Oberlin College.
Kagan, Benjamin M., Washington and Jefferson College.
Kaliss, Nathan, Student, Columbia University.
Kekwick, Ralph A., Visiting Fellow, Princeton University.
Keltch, Anna K., Research Assistant, Lilly Research Laboratories.
Kyle, John Arthur, Explorers Club, New York.
Landowne, Milton, Student, Harvard Medical School.
Lawlor, Sister Anna Catherine, Instructor in Biology, College of Saint Elizabeth.
Liedke, Kathe B., Student, Columbia University.
Marshak, Alfred, Student, Harvard University.
Marsland, Douglas A., Assistant Professor of Biology, Washington Square College, New York University.
Maxwell, Jane, Instructor in Biology, Carnegie Institute of Technology.
Mazia, Daniel, Graduate in Zoölogy, University of Pennsylvania.
Melampy, Robert M., Assistant in Animal Nutrition, Cornell University.
Menkin, Miriam F., Research Fellow in Pathology, Harvard Medical School.
Metzner, Jerome, Graduate Student, Columbia University.
Miller, James A., Graduate Assistant in Zoölogy, The University of Chicago.
Morris, Samuel, Instructor, Temple University.
Moser, Floyd, Graduate Student, University of Pennsylvania.
Nichol, Margaret A., University of Pittsburgh.
Novikoff, Alex Benjamin, Fellow, Brooklyn College.
Rankin, John S., Assistant, Wesleyan University.
Rubenstein, Boris B., Assistant in Physiology, The University of Chicago.
Rubidge, Karyl W., Student, Vassar College.
Russell, W. Lawson, Fellow, The University of Chicago.
Sauer, Fred C., Assistant Professor, University of Kansas.
Schweitzer, Morton D., Assistant in Zoölogy, Columbia University.
Shapiro, Herbert, Graduate Student, Princeton University.
Specht, Heinz, Graduate Student, Johns Hopkins University.
Stone, Faith, Scholar, The University of Chicago.
Stricker, George J., Student, Yale University.
Stuart, Martha S., University of Pittsburgh.
Summers, Francis M., Student, Columbia University.
Townsend, Grace, Assistant, The University of Chicago.
Walker, Paul A., Graduate Assistant, Harvard University.
Webster, M. Dorothy, Dalhousie University.
Wichterman, Ralph, Graduate Student, University of Pennsylvania.
Wilson, Hildegard, Assistant, New York University.

Research Assistants

Anthony, Genevieve, University of Pennsylvania.
Armstrong, Louise S., Research Assistant, Cornell University Medical College.

Auerbuck, Sylvia, Student, College of the City of New York.
Baron, Harry, Assistant Instructor, New York University.
Black, Peter, Student, McGill University.
Brown, William R., Graduate Student, University of Cincinnati.
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Coghill, Muriel, Fallsington, Pennsylvania.
Corson, Samuel A., Research Assistant, University of Pennsylvania.
Denny, Martha, Radcliffe College.
Doyle, William L., Brooks Fund Fellow, Johns Hopkins University.
Engel, Frank L., Student, Dartmouth College.
Engel, George L., Undergraduate, Dartmouth College.
Fisher, Kenneth C., Fellow in Department of Physiology, University of Toronto.
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Grand, C. G., Research Assistant, New York University.
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Harnly, Marie L., Assistant in Biology, New York University.
Hill, Edgar S., Research Assistant, Rockefeller Institute.
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Richardson, Margaret S., Cornell University Medical College.
Robertson, Lola Ellis, Research Assistant, New York University.
Rusch, Elizabeth, Research Assistant, Memorial Hospital.
Sell, James P., Graduate Assistant, Yale University.
Shaw, Isidor, Research Assistant, Long Island University.
Smith, Charles E., III, Medical Student, University of Pennsylvania.
Starkey, William F., Graduate Assistant, University of Pittsburgh.
Stein, Martin H., Student, Cornell University Medical College.
Stuart, Richard R., Graduate Assistant, State University of Iowa.
Wade, Lucille, Research Assistant, Lilly Research Laboratories.
Wallace, Edith M., Scientific Artist, Carnegie Institution of Washington.
Zujko, Alphonse J., Research Assistant, Trinity College.

Students

BOTANY

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Ramey, Sally, Student, Elmira College.

EMBRYOLOGY

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Armack, Clifford M., Curator of Biology, Museum of Northern Arizona.
Bates, M. Noble, Graduate Assistant in Zoölogy, Oberlin College.

Bell, Ruth, Wellesley College.
Bengel, Walter Z., Assistant in Anatomy and Embryology, DePauw University.
Chen, I., Graduate Student, University of Pennsylvania.
Churney, Leon, Graduate Student, University of Pennsylvania.
DeWolf, Robert A., Instructor in Zoölogy, Rhode Island State College.
Foster, Edith F., Undergraduate Student, Vassar College.
Godwin, Melvin C., Assistant in Histology and Embryology, Cornell University.
Creco, Filomena M., Hunter College.
Hamilton, Mary Alice, Elmira College.
Hooper, Kathryn T., Wheaton College.
Kagan, Benjamin M., Washington and Jefferson College.
Kriete, Frederic M., Student, DePauw University.
Lippman, Richard W., Yale University.
McAuley, Auley A., Student, DePauw University.
McGehee, Elise, Newcomb College, New Orleans.
Moser, Floyd, Graduate Student, University of Pennsylvania.
Root, Charlotte M., Ohio Wesleyan University.
Rose, Sylvan M., Graduate Assistant, Amherst College.
Rubidge, Karyl W., Student, Vassar College.
Spangler, Elizabeth A., Wheaton College.
Taylor, Hoyt C., Graduate Assistant. Wesleyan University.
Tukey, Gertrude R., Undergraduate, Smith College.
Turner, Robert S., Instructor, Dartmouth College.
Wardwell, Judith S., Laboratory Assistant, Wellesley College.
Zinn, Donald J., Student, Harvard University.

PHYSIOLOGY

Alt, Howard L., Associate in Medicine, Northwestern University Medical School.
Botsford, E. Frances, Assistant Professor of Zoölogy, Connecticut College.
Derrickson, Mary B., Frederica, Delaware.
Glassmeyer, Elmer J., Graduate Student, University of Cincinnati.
Havey, Clinton B., Acadia University.
Hibbard, Jeanne, Oberlin College.
Howell, Charles D., Johns Hopkins University.
Hull, Frank M., Head of Department of Physiology, University of Mississippi.
Johnson, Edna L., Associate Professor of Biology, University of Colorado.
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Ross, Edward, Student, University of California.
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Solandt, Omond McK., Research Assistant, University of Toronto.
Stricker, George J., Undergraduate, Yale University.
Vexler, Darwin E., Student, Rutgers University.
Webster, Margaret D., Dalhousie University.

PROTOZOÖLOGY

Bechtel, Wilmer R., Columbia University.
Boyer, Dade C., Douglas School.
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Hirschfeld, Nathan B., Columbia University.
Moore, Raymond P., West Virginia State College.
Orbison, Agnes M., Professor of Biology, Elmira College.
Perkins, Irene T., Teacher of Biology, Columbia University.

Stubbs, Trawick H., Instructor in Biology, Emory Junior College.
Urban, John, Columbia University.
Ward, Mary, Wellesley College.
Young, Martin D., Instructor in Biology, Junior College of Augusta.

INVERTEBRATE ZOÖLOGY

Abramowitz, Alexander A., Graduate Student, Harvard University.
Bartholomew, Olive F., Radcliffe College.
Blades, Helen N., Teacher, University of Michigan.
Blagg, Amy Elizabeth, Assistant Professor of Zoölogy, Grinnell College.
Bosworth, Millard W., Assistant in Biology, Wesleyan University.
Bowman, Sarah B., Assistant, Agnes Scott College.
Brooks, Virginia C., Student, Wilson College.
Buck, John B., Assistant in Zoölogy, Johns Hopkins University.
Buell, Katherine M., Oberlin College.
Carmack, Ted, Student Assistant, Wabash College.
Clark, Frances J., Student, University of Rochester.
Collings, William D., Undergraduate Laboratory Assistant, DePauw University.
Cowles, Janet M., Johns Hopkins University.
Denny, Martha, Student, Radcliffe College.
DiPaola, Rose M., Hunter College.
Field, Mary F., Laboratory Assistant, Brearley School.
Gaw, Harry, Student, Yale University.
Giddings, W. Philip, Student, Amherst College.
Henderson, Allen R., Student, Yale University.
Horton, Richard G., Student, Williams College.
Hunter, Francis R., California Institute of Technology.
Jones, Leo M., Undergraduate Laboratory Assistant, DePauw University.
Jones, Roy W., Associate Professor of Biology, Oklahoma University.
Kelly, Florence C., Instructor, Simmons College.
Lagler, Karl F., University of Rochester.
Livingston, Mary C., Student Assistant, American University.
Mast, Louise R., Graduate Student, Johns Hopkins University.
Metcalf, Isaac S. H., Undergraduate, Oberlin College.
Miller, Thomas R., Undergraduate, Hamilton College.
Nichols, Ray J., Graduate Student, University of Illinois.
Odell, Floyd A., Assistant in Zoölogy, Yale University.
Painter, Ben T., Instructor in Zoölogy, College of William and Mary.
Parker, Rachel W., Goucher College.
Podolsky, Sophia, Goucher College.
Rogers, Philip V., Instructor, Hamilton College.
Rohm, Pauline B., Oberlin College.
Shaw, Ruth K., Student, Mount Holyoke College.
Shepard, Margaret J., Graduate Assistant, Mt. Holyoke College.
Smith, Stuart D., Student, Wabash College.
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Taylor, Hoyt C., Graduate Assistant in Biology, Wesleyan University.
Todd, Robert E., Jr., Harvard University.
Trezise, Willard J., Assistant in Zoölogy, Johns Hopkins University.
Van Deventer, William C., Graduate Assistant, University of Illinois.
Wharton, Marguerite, Student Assistant, New Jersey State Teachers College.
Wing, Lucius T., Undergraduate, Harvard University.

Whittinghill, Maurice, Instructor, Dartmouth College.
Williams, Inez W., Massachusetts State College.
Williams, Margarita, University of Iowa.
Woodside, Gilbert, Assistant in Zoölogy, Harvard University.

3. TABULAR VIEW OF ATTENDANCE

	1929	1930	1931	1932	1933
Investigators—Total	329	337	362	314	319
Independent	234	217	236	212	210
Under Instruction	71	87	83	73	66
Research Assistants	24	33	43	29	43
Students—Total	125	136	125	132	118
Zoölogy	53	56	55	55	54
Protozoölogy	15	14	17	16	11
Embryology	28	27	29	29	28
Physiology	17	23	17	18	19
Botany	12	16	7	14	6
Total Attendance	454	473	487	446	437
Less Persons registered as both students and investigators	10	14	20	14	12
	444	459	467	432	425
Institutions Represented—Total	123	126	137	141	120
By Investigators	96	95	102	94	92
By Students	64	71	68	76	58
Schools and Academies Represented					
By Investigators	—	—	—	—	1
By Students	1	4	4	1	2
Foreign Institutions Represented					
By Investigators	30	7	8	8	5
By Students	3	2	1	1	—

4. SUBSCRIBING AND COÖPERATING INSTITUTIONS, 1933

American University
Amherst College
Atlanta University
Bowdoin College
Bryn Mawr College
Chinese Educational Mission
College of St. Elizabeth
Columbia University
Commonwealth Fund
Cornell University
Cornell University Medical College
C. R. B. Foundation
De Pauw University
Duke University
General Education Board
Goucher College
Hamilton College
Harvard University
Harvard University Medical School
Hunter College
Industrial & Engineering Chemistry, of the American Chemical Society
Johns Hopkins University

Johns Hopkins University Medical School
Eli Lilly & Co.
Long Island University
Memorial Hospital of N. Y. City
Morehouse College
Mount Holyoke College
Museum of Northern Arizona
New York State Department of Health
New York University
New York University Medical School
Oberlin College
Pennsylvania College for Women
Princeton University
Radcliffe College
Rockefeller Foundation
Rockefeller Institute for Medical Research
Rutgers University
Smith College
Sophie Newcomb College
Swarthmore College
Syracuse University
Tufts College
University of Chicago
University of Cincinnati
University of Iowa
University of Pennsylvania
University of Pennsylvania Medical School
University of Pittsburgh
University of Rochester
University of Rochester Medical School
University of Wisconsin
Vanderbilt University Medical School
Vassar College
Wabash College
Wellesley College
Wesleyan University
West Virginia State College
Wheaton College
Williams College
Wilson College
Wistar Institute of Anatomy and Biology
Yale University

5. EVENING LECTURES, 1933

Friday, July 7
Dr. G. H. Parker .... "Transmission of Neurohumoral Substances."

Tuesday, July 11
Dr. The Svedberg .... "Ultracentrifugal and Cataphoretic Studies on Respiratory Proteins."

Friday, July 21
Dr. Balduin Lucké .... "The Zoölogical Distribution of Tumors."

Friday, July 28
Dr. Laurence Irving .... "On the Ability of Mammals to Survive Without Breathing."

Wednesday, August 2
Dr. Oscar E. Schotté .... "Organizers and Inherent Potencies in the Embryonic Development of Amphibians."

Friday, August 4
Dr. August Krogh .... "Conditions of Life in the Depths of the Ocean."

Friday, August 11
Dr. F. Spek .... "Die Protoplasmadifferenzierung der Eizellen während der ersten Entwicklung."

Friday, August 18	
Dr. John H. Northrop	"Evidence of the Protein Nature of Pepsin and Trypsin."
Friday, August 25	
Dr. Edwin Grant Conklin	"Science and Progress."
Friday, September 1	
Dr. Robert Keith Cannan	"Studies in the Amphoteric Properties of Proteins."

SPECIAL LECTURES AND MOTION PICTURES

Saturday, July 1	
Dr. A. V. Hill	"Nerve."
Monday, July 17	
Dr. Robert Chambers	Motion pictures of studies on the insemination and segmentation of marine ova.
Thursday, August 24	
Dr. Robert Chambers and Mr. C. G. Grand	"Tissue Culture Technique and Various Aspects of the Growth of Normal and Cancerous Tissues."

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6. SHORTER SCIENTIFIC PAPERS, 1933

Friday, June 30	
Dr. Ethel B. Harvey	"Effects of Centrifugal Force on the Ectoplasmic Layer, Nuclei, and Protoplasm of Fertilized Sea Urchin Eggs."
Dr. E. Newton Harvey	Motion pictures taken through the centrifuge-microscope illustrating differences in behavior of unfertilized and fertilized sea urchin eggs.
Dr. William C. Young	"Some Data from a Correlated Anatomical, Physiological, and Behavioristic Study of the Reproductive Cycle in the Female Guinea Pig."
Dr. William R. Amberson	"Hæmoglobin-Ringer, a New Mammalian Perfusion Fluid."
Wednesday, July 5	
Dr. G. W. Prescott	"Some Effects of Blue-green Algæ on Lake Fish."
Dr. Hugh P. Bell	"Distribution and Ecology of the Marine Algæ of the Maritime Provinces of Canada."

Dr. W. R. Taylor .... “Distribution of Newfoundland Algæ.”

Monday, July 10

Dr. A. C. Redfield .... “The Concentration of Organic Derivatives in Sea Water, in Relation to the Chemical Composition of Plankton.”

Dr. George L. Clarke .... “Diurnal Migration of Plankton in the Gulf of Maine and Its Correlation with Changes in Submarine Irradiation.”

Dr. Selman A. Waksman and
Miss Cornelia L. Carey .... “The Rôle of Bacteria in the Formation of Nitrate in the Sea.”

Friday, July 14

Dr. M. M. Brooks .... “The Effect of Respiratory Poisons and Methylene Blue on Cleavage of Certain Eggs.”

Dr. Laurence Irving and
Miss Jeanne F. Manery .... “Ionic Changes During the Development of Fish Eggs.”

Dr. E. Newton Harvey .... “The Tension at the Surface of Egg Cells.”

Mr. Donald P. Costello .... “Fertilization Membranes of Centrifuged Asterias Eggs.”

Dr. Robert Chambers .... “The Kidney Tubules with Phenol Red and Neutral Red.”

Tuesday, July 18

Dr. G. W. Kidder .... “Chromatin Extrusion in Certain Ciliate Commensals of Mussels.”

Mr. F. M. Summers .... “The Reorganization Bands in the Macronucleus of Aspidisca.”

Mr. Harold E. Finley .... “Comparative Studies on the Osmiophilic and Neutral-Red-Stainable Inclusions of the Genus Vorticella.”

Mr. William L. Doyle .... “Experimental Cytology of Amœba proteus.”

Tuesday, July 25

Dr. H. B. Goodrich and
Mr. C. B. Crampton .... “One Step in the Development of Hereditary Pigmentation in the Fish Oryzias latipes.”

Dr. George D. Snell .... “Translocations in the Mouse and Their Effect on Development.”

Dr. D. E. Lancefield .... “A Series of Probable Mutations in Drosophila pseudo-obscura as Compared with D. melanogaster.”

Dr. P. W. Whiting .... “Sex-determination in Hymenoptera.”

Tuesday, August 1	
Dr. Conway Zirkle	"The Effects of Fat Solvents Upon the Fixation of Mitochondria."
Dr. Arthur W. Pollister	"The Cytology of Amphibian Tissues."
Dr. B. M. Duggar and Dr. Alexander Hollaender	"The Irradiation of Biological Suspensions by Monochromatic Light. (The effect of ultra-violet light on a plant virus and bacteria.)"
Dr. C. C. Speidel	Motion pictures showing some varieties of nerve irritation, as seen in living frog tadpoles.
Tuesday, August 8	
Dr. Edmund K. Hall	"Regional Differences in the Organization Center of the Amphibian Embryo."
Dr. George A. Baitsell	"Observations on Migrating Cells in Cultures of Amphibian Tissues, Particularly with Reference to the Problem of Fiber Formation."
Dr. V. Hamburger	"The Effect of Wing Bud Extirpations in Chick Embryos on the Development of the Nervous System."
Dr. G. H. Parker	"Color Changes in the Dogfish."
Thursday, August 10	
Dr. A. B. Dawson	"The Absorption of Colloidal Carbon by the Mesonephric Epithelium of Necturus."
Mr. Victor Schechter	"Morphological and Electrophoretic Effects of the Galvanic Current on Griffithsia Cells."
Mr. K. Dan	"The Electric Charge on the Surface of Sea Urchin Eggs."
Dr. R. W. Gerard	"Electrical Activity of the Brain."
Tuesday, August 15	
Dr. A. H. Palmer	"The Isolation of a Crystalline Globulin from the Albumin Fraction of Cow's Milk."
Dr. Paul Reznikoff	"Studies in Iron Metabolism in Humans."
Dr. Marie Krogh	"The Hormonal Connection between the Pituitary and the Thyroid."
Dr. F. E. Chidester	"Anterior Pituitary Like Hormone Effects."
Tuesday, August 22	
Mr. R. Rugh	"Heterochromatic Radiation and Early Amphibian Development."

Dr. R. E. Zirkle	"A Non-linear Relation Between Biological Effect and Ionizing Power of Alpha Rays."
Dr. Leon C. Chesley	"Effects of X-rays Upon Cell Oxidations."
Dr. P. S. Henshaw and Dr. D. S. Francis	"A Response of Arbacia Eggs to X-rays."
Tuesday, August 29	
Dr. Charles B. Wilson	"The Copepod Plankton of the Last Cruise of the Non-magnetic Ship 'Carnegie.'"
Dr. Dugald E. S. Brown	"The Pressure Coefficient of Viscosity in the Eggs of Arbacia."
Dr. Edwin P. Laug	"Observations on Lactic Acid, Total CO_2, and pH of Venous Blood During Recovery from Severe Exercise."
Dr. Anna R. Whiting	"A Study of Eye Color in the Parasitic Wasp, Habrobracon."
Dr. Marc A. Graubard	"The Melanin Reaction in Races of Drosophila melanogaster."
Thursday, August 31	
Dr. Paul A. Reznikoff and Mrs. Dorothy G. Reznikoff	"Blood Cell Studies in Dogfish."
Dr. W. H. F. Addison	"Intracranial Pigmentation in Teleosts."
Dr. E. R. Clark and Mrs. Eleanor Linton Clark	"The Blood Capillary in Relation to Contractility."
Mr. Herbert L. Eastlick	"Striated Muscles of the Lamellibranch Mollusc, Pecten gibbus."
Dr. Arthur W. Pollister	"The Centrioles of Amphibian Tissues."
Mr. Theodore G. Adams	"The Chromidium in Arcella vulgaris."
Dr. Harold H. Plough	"Selective Fertilization in Styela."
Mr. Meyer Atlas	"Relation of Temperature and Cleavage in Frogs' Eggs."
Dr. E. G. Conklin	"Disorientations of Development in Crepidula, Caused by Cold."
Dr. Ethel Browne Harvey	"Changes in the Arbacia Egg Immediately Following Fertilization, as Determined by Centrifugal Force."
Dr. P. S. Henshaw and Dr. D. S. Francis	"Recovery from X-ray Effects Before Fertilization in Arbacia Eggs and Its Effect on Development."

Miss Anna K. Keltch,
Miss Lucille Wade and
Dr. G. H. A. Clowes .... "Further Observations on the Contrasting Sensitivity of Eggs and Sperm to Various Chemical Agents."

Dr. G. H. A. Clowes,
Miss Anna K. Keltch and
Miss Lucille Wade .... "Variations in the Sensitivity of Eggs Following Fertilization."

Dr. E. Newton Harvey .... "Flattening of Marine Eggs Under the Influence of Gravity."

Dr. L. V. Heilbrunn .... "The Action of Anæsthetics on the Surface Precipitation Reaction."

Dr. Dorothy R. Stewart and
Dr. M. H. Jacobs .... "The Effect of Certain Salt Solutions on the Permeability of the Arbacia Egg."

Dr. B. R. Speicher .... "The Effective Period in Development of the Mutant Factor 'Eyeless' in Habrobracon."

Dr. Anna R. Whiting .... "Variegated Eye Color in Habrobracon."

Dr. P. W. Whiting .... "Egg-trinuclearity in Habrobracon."

Mr. Heinz Specht .... "Relation Between Oxygen Tension and Respiration in Spirostomum ambiguum, with Corrections for Ammonia."

Dr. C. S. Shoup .... "Respiration and Luminescence of Bacteria in Carbon Monoxide."

Dr. G. Wellford Taylor .... "The Relation Between Luminescence and Respiration in Bacteria with Especial Reference to the Effects of Narcotics."

Dr. Lyle V. Beck .... "Nature of the Aerobic Apparent Reduction Potential."

Dr. Eric G. Ball .... "The Relative Abundance of Hydrogen Isotopes in Sea Water."

Dr. Oscar W. Richards .... "Toxicity of Some Metals and Berkefeld Filtered Sea Water to Mytilus Edulis."

Dr. Herbert H. Jasper .... "Some New Aspects of the Physiology of the Nerve-Muscle System in Crustacea Brought Out by Electrical Excitation and Response."

Dr. Margaret Sumwalt and
Miss Kathryn McLane .... "The Blood Pressure of Limulus."

Mr. John C. Bridges and
Dr. Margaret Sumwalt .... "The Effect of pH Upon Potassium Penetration into Fundulus Eggs."

Dr. Arthur K. Parpart	"A Method for Following Volume Changes of Cells."
Dr. William R. Amberson, Mr. Frank Engel, Miss Dorothy Webster and Dr. Edwin P. Laug	"The Influence of pH upon the Passage of Hæmoglobin Through the Glomerulus of the Perfused Frog's Kidney."
Dr. M. H. Jacobs and Dr. Arthur K. Parpart	"The Influence of the Escape of Salts on the Osmotic Behavior of the Erythrocyte."

7. MEMBERS OF THE CORPORATION

1. Life Members

Allis, Mr. E. P., Jr., Palais Carnoles, Menton, France.
Andrews, Mrs. Gwendolen Foulke, Baltimore, Md.
Billings, Mr. R. C., 66 Franklin St., Boston, Mass.
Conklin, Prof. Edwin G., Princeton University, Princeton, N. J.
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Foot, Miss Katherine, Care of Morgan Harjes Cie, Paris, France.
Gardiner, Mrs. E. G., Woods Hole, Mass.
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Jackson, Mr. Chas. C., 24 Congress St., Boston, Mass.
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King, Mr. Chas. A.
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Minns, Miss Susan, 14 Louisburg Square, Boston, Mass.
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Morgan, Mrs. T. H., Pasadena, Calif.
Noyes, Miss Eva J.

Osborn, Prof. Henry F., American Museum of Natural History, New York, N. Y.

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Shedd, Mr. E. A.

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Trelease, Prof. William, University of Illinois, Urbana, Ill.

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Wilson, Dr. E. B., Columbia University, New York City, N. Y.

2. Regular Members, 1933

Adams, Dr. A. Elizabeth, Mount Holyoke College, South Hadley, Mass.

Addison, Dr. W. H. F., University of Pennsylvania Medical School, Philadelphia, Pa.

Adolph, Dr. Edward F., University of Rochester, School of Medicine and Dentistry, Rochester, N. Y.

Allee, Dr. W. C., University of Chicago, Chicago, Ill.

Allyn, Dr. Harriet M., Mount Holyoke College, South Hadley, Mass.

Amberson, Dr. William R., University of Tennessee, Memphis, Tenn.

Anderson, Dr. E. G., California Institute of Technology, Pasadena, Calif.

Armstrong, Dr. Philip B., Cornell University Medical College, 1300 York Avenue, New York City, N. Y.

Austin, Dr. Mary L., Wellesley College, Wellesley, Mass.

Baitsell, Dr. George A., Yale University, New Haven, Conn.

Baldwin, Dr. F. M., University of Southern California, Los Angeles, Calif.

Ball, Dr. Eric G., Johns Hopkins Medical School, Baltimore, Md.

Bard, Prof. Philip, Johns Hopkins Medical School, Baltimore, Md.

Barron, Dr. E. S. Guzman, Department of Medicine, The University of Chicago, Chicago, Ill.

Barth, Dr. L. G., Columbia University, New York City, N. Y.

Beckwith, Dr. Cora J., Vassar College, Poughkeepsie, N. Y.

Behre, Dr. Elinor H., Louisiana State University, Baton Rouge, La.

Bennitt, Dr. Rudolf, University of Missouri, Columbia, Mo.

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Bigelow, Prof. R. P., Massachusetts Institute of Technology, Cambridge, Mass.

Binford, Prof. Raymond, Guilford College, Guilford College, N. C.

Bissonnette, Dr. T. H., Trinity College, Hartford, Conn.

Blanchard, Prof. Kenneth C., New York University, Washington Square College, New York City, N. Y.

Bodine, Dr. J. H., University of Iowa, Iowa City, Ia.

Boring, Dr. Alice M., Yenching University, Peking, China.

Bowling, Miss Rachel, Columbia University, New York City, N. Y.

Box, Miss Cora M., University of Cincinnati, Cincinnati, O.

Bradley, Prof. Harold C., University of Wisconsin, Madison, Wis.

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Bronk, Dr. D. W., University of Pennsylvania, Philadelphia, Pa.

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OBSERVATIONS ON THE ERYTHROPHORES OF SCORPÆNA USTULATA

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Among those fishes most commonly used in studies concerned with color changes the dominant pigment-bearing cell has been the melanophore, and as a consequence the most obvious chromatic responses in these animals have been conditioned largely by alterations in pigment distribution within these melanophores. Inevitably this has attracted more attention to this type of cell than to the xanthophores and erythrophores even though these yellow and red-bearing pigment cells might also play a part in the color changes of such fishes. There is, however, a fair amount of literature dealing with the reactions of erythrophores and xanthophores, enough to raise some interesting questions as to their control and behavior. A goodly part of this work has been devoted to a determination of the governing factors, particularly humoral, responsible for the contribution of the erythrophores to the nuptial coloration displayed by certain forms during their spawning season.

In the scorpian fish, *Scorpæna ustulata,* the dominant pigment-bearing cell is the erythrophore. Since this form could be obtained in numbers suitable for our purpose, a particularly favorable opportunity was given us to study erythrophore activity while at the Naples Zoological Station, where this investigation was done in the autumn of 1931. We wish to take this opportunity to express our appreciation for the privilege of using the Columbia-Woods Hole table at this institution, and also to express our gratitude and thanks to Professor R. Dohrn, the Director of the Station, for the courtesies which he extended to us during our stay in Naples.

As usually found in and about the waters of the Bay of Naples, the animal has a deep red color over the entire dorsal and lateral surface of the body, due to the presence of large numbers of erythrophores in the scales and in the deeper layers of the skin. When a scale is removed from the fish and examined microscopically, the plentitude of erythrophores is apparent, melanophores and xanthophores being scarce in comparison. Realizing the importance of erythrophores in contributing to the color of many tropical and sub-tropical fishes where the dominating tone is frequently red, it was thought desirable to investigate the behavior of these cells in *Scorpæna ustulata*, both in the intact animal and in isolated scale preparations. Whenever convenient, observations were also made on the xanthophores and melanophores.

EXPERIMENTAL

The animals used in these experiments were all adults 20 to 30 cm. in length. When kept upon a black background they have a brilliant red color, but when placed upon white, this color fades to a pale pink. This fading requires anywhere from several hours to over a day to come to completion, a relatively slow process when compared with the rapidity of color changes in some other fishes. Similarly, when returned to black, the resumption of the deep red takes an equally long time. This raises the question as to whether or not these color changes are humorally controlled in the sense described by Giersberg (1930) for *Phoxinus,* where there is no evidence of a nervous control over the erythrophores. In *Scorpæna,* however, such evidence is not lacking, for if the sympathetic cord be severed at a point midway on the trunk in an animal adapted to a dark background and the fish be placed upon a white background, the portion of the body posterior to the cut retains its characteristic deep red color even after the portion anterior to the cut has paled. Such an experiment is of the type originally done by Pouchet (1876) and v. Frisch (1911, 1912) in demonstrating nervous control of the chromatophores. Furthermore, if the anterior end of the *Scorpæna* medulla be electrically stimulated when the animal is deep red, the whole body immediately pales, but in an animal where the sympathetics in the posterior trunk have been sectioned, stimulation of the medulla produces paling only in the skin anterior to the cut. The paling in all cases occurs with great rapidity (10 to 15 seconds) in marked contrast to its slow development when the fish is placed on a white background. There is then ample reason to believe that in *Scorpæna* the erythrophores not only possess nervous connections with a pigmentary motor-center in the medulla, but they are also capable of receiving and responding to impulses sent out from this center. The slowness with which the response to background changes develops is suggestive of some sort of neuro-humoral control of the type described by Parker (1932).

The presence of functional nervous connections in the erythrophores of *Scorpæna* places them in a group with those of *Crenilabrus pavo* and *Trigla corax.* These two forms, according to v. Frisch (1912), possess erythrophores and xanthophores with an innervation similar to that of the melanophores, as judged by the effects of electrical stimulation of the pigment-motor center in the medulla. In *Scorpæna*, *Crenilabrus*, and *Trigla* the impulses are distributed to the chromatophores by way of the sympathetic system. Lode (1890), however, early reported the failure of the erythrophores of the trout to contract when the spinal cord or medulla were stimulated electrically, although such stimulation produced melanophore contraction. It was only on prolonged local stimulation that he observed any contraction of the erythrophores. Furthermore, Giersberg (1930) could find no evidence for a nervous control of the erythrophores and xanthophores of *Phoxinus*, the usual tests for nervous control such as stimulating the pigment-motor center in the medulla or cutting the sympathetics failing to evoke any positive response. Later Giersberg (1932) demonstrated that extirpation of the hypophysis in *Phoxinus* prevents all responses on the part of the erythrophores and xanthophores, thus giving added weight to his conclusion that erythrophore and xanthophore activity in *Phoxinus* was controlled solely through the activity of this gland. Fries (1931), working on *Fundulus heteroclitus*, a form possessing no erythrophores, demonstrated some measure of nervous control of the xanthophores, but he also concluded, in view of the differences in response between the xanthophores and melanophores, that a humoral mechanism of some sort was involved, acting to enforce the nervous mechanism or even at times possibly superceding it. Matthews (1933), however, observed in *Fundulus* the usual responses of the xanthophores to white and yellow background after hypophysectomy. In his operated animals the xanthophores contracted over white and expanded over yellow, as did those of normal unoperated fishes. The possibility of two different controlling mechanisms being present in the fishes in respect to lipophore activity, as suggested by Giersberg (1932), one nervous and the other humoral, is certainly worthy of serious consideration. In all probability a third type must also be considered, one where both mechanisms are present, one or the other assuming greater or less prominence as the case may be.

An extremely interesting phenomenon which many pigment cells exhibit under certain circumstances is the ability to show rhythmical pulsations after the proper treatment. Such pulsations, consisting as they do of a regular migration of the pigment granules in and out of the processes, were first observed by Ballowitz (1913*b*) in the erythrophores of excised skin from the trunk of *Mullus* when immersed in 0.75 per cent NaCl. This phenomenon is undoubtedly of the same nature as one described later by Spaeth (1916) in *Fundulus* melanophores. In this case isolated scales were immersed in N/10 $BaCl_2$ for about five minutes and then transferred to N/10 NaCl, where after a wait of fifteen minutes or so the scale melanophores showed pronounced rhythmical pulsations. Methods other than treatment with $BaCl_2$ have also been described as producing pulsations in *Fundulus* melanophores (Smith, 1930; Yamamato, 1933). Isolated scale erythrophores of *Scorpæna*, when placed in N/10 NaCl, expand and remain so for about two to three hours until they begin to contract, resembling in this respect the melanophores of *Fundulus*. Furthermore if, again like the melanophores of *Fundulus*, the scales are first immersed in $BaCl_2$ and left there until all of the erythrophores are either completely or almost completely contracted (which usually occurs in about five minutes) and then returned to N/10 NaCl, the erythrophores will shortly begin a series of regular pulsations lasting for two or three hours. These pulsations seem to be of the sort described by Spaeth in *Fundulus* melanophores and probably, as is also the case in *Fundulus* melanophores, differ only from those seen by Ballowitz in *Mullus* in that the movements do not start spontaneously in NaCl.

Effects of Drugs on Isolated Scale Erythrophores

Isolated scale material from *Scorpæna* was found extremely well adapted for the study of the effects of drugs upon erythrophores. The scales lend themselves particularly well to such a study as they are easily removed from the body, are sufficiently transparent for microscopic work, and are of a favorable size, ranging from 2 to 4 mm. in width. Spaeth (1913) was the first to recognize the usefulness of such preparations in making direct microscopic observations upon the response of the chromatophores to various stimuli.

In testing the effects of drugs upon the isolated scale erythrophores of *Scorpæna*, the following procedure was adopted. First the scales were immersed in a solution made up of six parts of N/10 NaCl and one part N/10 KCl. Such a solution might or might not produce and maintain a condition of slight erythrophore expansion. If not, more NaCl or KCl was added to the solution, NaCl if the erythrophores were contracted, KCl if they were expanded, until a mixture was obtained which did maintain the erythrophores in the stellate state within the time limits of the experiment. Having attained a suitable combination of NaCl and KCl, the drug to be tested was added to the mixture to make whatever concentration was desired, a certain portion of the original having been set aside previously as a control. From time to time the condition of the erythrophores in the experimental solution was compared with that of the cells in the control. Such a procedure, while not entirely satisfactory, was necessary as the combined effects of NaCl and KCl were variable within certain prescribed limits. But inasmuch as the drugs to be tested had powerful effects in either one direction or the other, the method was found suitable in determining whether a drug acted either to expand or contract the chromatophore. In any discussion of the changes in pigment distribution within the chromatophore it must be borne in mind that the pigment granules migrate in and out of permanently formed cellular processes (Ballowitz, 1913*a*; Matthews, 1931).

Nicotine

Scales with stellate erythrophores immersed in nicotine (Merck) solutions ranging from 1/100,000 to 1/10,000 showed first an immediate and pronounced expansion of all erythrophores. Eventually this expansion was replaced by the contracted or stellate condition, usually in about twenty minutes. This contraction persisted for at least three hours, the longest period that any single case was kept under observation. Between concentrations of 1/10,000 to 1/2,000 there was first a slight expansion (in one case at 1/2,000 no expansion whatever) which was followed in about twenty minutes by complete and lasting contraction. With stronger solutions (1/1,000–1/100) the higher concentration produced an immediate expansion which persisted without change, the lower concentrations (1/1,000) also leading to the same result although not so rapidly.

The initial effect then, regardless of strength, was one of expansion, followed in the case of weaker solutions by a contraction. This order, peculiarly enough, is just the reverse of what is obtained when nicotine is injected into the fish, such injections giving first a contraction, due presumably to an initial stimulation of the sympathetic ganglia, followed by an expansion, the result of an eventual peripheral sympathetic paralysis. Why the order should be reversed on the immersion of isolated scale erythrophores in nicotine solutions and whether the reversal is of any significance is difficult to say.

Physostigmine

Physostigmine acts to produce an expansion of the erythrophore pigment, an expansion which persists for at least one and one-half to two hours, the longest time any one scale was kept under observation. This expansion was observed in 0.0065 per cent and 0.013 per cent solutions. No other concentrations were tried. The expansion was at its maximum usually within ten or fifteen minutes after immersion. In these so-called expanded cells the pigment distribution in most cases presented certain peculiarities, the pigment as a rule being mostly concentrated at the ends of the processes and in the central body. Since those parts of the processes between the tip and central body were filled only with a few scattered granules, the cell had the appearance of a central pigment mass surrounded by an outer pigment ring with an apparent empty space between. Close inspection, however, revealed sufficient pigment within the processes to outline them. But the appearance of the cells differed markedly from that presented when they were immersed in NaCl. In such a salt the pigment is evenly distributed throughout the processes and central body. "Ringed" erythrophores, as seen in the physostigmine solutions, when placed in a mixed NaCl and KCl solution again became stellate and when put in KCl immediately contracted. Such contracted erythrophores when transferred to NaCl expanded in the usual manner, the previous exposure to physostigmine in no way preventing a subsequent even distribution of the pigment. Not all of the scales placed in physostigmine showed the "ringed" type of expansion just described. In some cases the expansion appeared to be of the usual type with the pigment evenly distributed in the processes. In others the amount of pigment in the processes was relatively scant and the process consequently had a thin frayed-out look quite different from the well-defined and well-filled-out appearance shown in NaCl. In their responses to physostigmine, the melanophores were about the same as the erythrophores, except that as a rule the melanophore expansion tended to be better defined, less thin and less frequently "ringed."

Pilocarpine

Pilocarpine in concentrations ranging from 0.016 per cent to 0.04 per cent had essentially the same effects as physostigmine, producing in the majority of cells a thin or "ringed" expansion of both the erythrophores and the melanophores.

Atropine

Atropine in concentrations ranging from 0.025 per cent to 0.0065 per cent produced an immediate expansion of the erythrophores and melanophores which was rapidly converted into the "ringed" expansion of the type already described under physostigmine. The second effect appeared within two to ten minutes after the exposure to the drug and persisted for as long as the scales were left in the solution. When such atropinized scales were transferred to N/10 KCl within thirty to forty minutes after their initial immersion in atropine, an interesting reversal of the usual response on the part of the chromatophores to KCl was seen. Ordinarily N/10 KCl immediately contracts and maintains contracted all of the chromatophores of isolated *Scorpæna* scales for at least two hours. Atropinized scales immersed in N/10 KCl showed the usual immediate contraction of their erythrophores and melanophores, but instead of staying contracted for an extended period as would be normally expected in such cases, these chromatophores quickly reversed their condition and rapidly assumed the state of maximal expansion, remaining so for at least two hours. This expansion usually occurred within two minutes after the transfer from atropine, although treatment with the weakest effective solutions of this drug required longer times—five minutes or so—before the re-expansion was established. In all scales the re-expanded erythrophores showed processes in which the pigment was evenly distributed. Control scales treated in precisely the same manner as the atropinized ones—except for the treatment with atropine—contracted in the usual manner in N/10 KCl, and stayed contracted for the usual two hours or so. We have then a clear and well-established case of the reversal of the effect of KCl brought on by previous treatment with atropine. To the best of our knowledge such a reversal has never been reported before. As mentioned previously, the reactions of erythrophores and melanophores were alike. The specificity of the effect as far as KCl is concerned is shown by the fact that when the scales are immersed in 1/100,000 adrenaline after atropine, the resulting contraction of the chromatophores is permanent. In other words, atropine does not reverse the effect of adrenaline, although it does reverse the effect of KCl. Atropinized chromatophores, expanded in KCl, when placed in 1/100,000 adrenaline immediately contracted and stayed contracted.

The atropine reversal of the effect of KCl is particularly interesting when considered in conjunction with the results of Spaeth and Barbour (1917), who found that adrenaline after ergotoxin produced an expansion instead of a contraction of the melanophores, a contraction which might or might not be lasting. The contracting effect of KCl, however, was unaffected, although its development was usually less rapid. In *Scorpæna*, however, the KCl effect upon the erythrophores and also upon the melanophores is reversed by atropine while the adrenaline effect is not changed. Spaeth and Barbour believed adrenaline to act upon the sympathetic endings in the chromatophore, while KCl acted upon the cell directly. Adrenaline apparently does act as they thought, but whether KCl is directly protoplasmic in its effect is another matter. In any event the results on *Scorpæna* erythrophores and on *Fundulus* melanophores show clearly that the contracting action of adrenaline and

KCl are not due to their action upon the same receptive mechanism within the cell.

The typical "ringed" expansion produced by atropine is also produced by pilocarpine and physostigmine and would indicate a direct protoplasmic effect upon the erythrophore rather than any action upon nerve endings present in the isolated scale preparations. In spite of this similarity in action, atropine is nevertheless separable from the other two drugs by virtue of its reversal of the effect of KCl, something which pilocarpine and physostigmine do not produce. Obviously atropine has some subtle action on the erythrophores different in nature from that of pilocarpine and physostigmine which is not disclosed by the character of the pigment distribution produced by these three drugs.

Acetyl-Choline

Solutions of acetyl-choline (0.1 per cent to 0.01 per cent) had no observable effect upon either the erythrophores or the melanophores of *Scorpæna ustulata.*

Cocaine

Cocaine in solutions of 0.1 per cent to 0.001 per cent produced contraction of the erythrophores and the melanophores in *Scorpæna ustulata.* There was, however, a pronounced tendency on the part of the melanophores to contract much more slowly than the erythrophores, the former contracting in about five minutes and the latter in about ten to twenty minutes, depending on the strength of the solution. These contractions lasted at least three hours. Scales with contracted chromatophores when returned to the N/10 NaCl–KCl mixture showed a re-expansion of their pigment cells to the stellate within twenty to thirty minutes. It is possible though not proven, that cocaine contracts the isolated scale erythrophores by virtue of its excitatory effect upon the peripheral sympathetic fibers.

Ethyl Alcohol

Scales with chromatophores in the stellate condition when placed in 10 per cent alcohol, usually show a pronounced expansion of all the pigment cells, the expansion occurring within the first few minutes after immersion. This in turn is replaced within the next five minutes by a concentration of the pigment within the central body. Within fifteen to thirty minutes, however, the pigment cells once more expand, this time to the "ringed" condition, in which they remain for at least three hours. In 5 per cent alcohol the same sequence of reactions is observed except perhaps that the final expansion is longer delayed, often not occurring until almost one hour after the beginning of the experiment. In 1 per cent alcohol, however, there is in the majority of the cases no sign of any initial expansion; instead the primary response of the chromatophores is a contraction, oftentimes immediate or on occasion delayed for fifteen or twenty minutes. Having once contracted in 1 per cent alcohol, the pigment cells remain in that condition for three hours at least, the longest time any set of experimental scales was kept under observation. In no case was any sign of expansion evident within this period. One scale with stellate erythrophores did show a slight expansion when the scale was first placed in 1 per cent alcohol, but this expansion in no way approached the maximum and was replaced within fifteen minutes by a complete contraction. In most cases in 1 per cent alcohol the erythrophores contracted before the melanophores, and when the time for re-expansion had come the melanophores usually expanded before the erythrophores.

The "ringed" expansion in the stronger alcoholic solutions is suggestive of protoplasmic injury as it is with pilocarpine, physostigmine, and atropine; but the primary expansion and subsequent contraction are probably not direct effects. The possibility of explaining these responses, and also the responses to nicotine, by assuming an effect on the endings of two different types of nerve fibers within the erythrophores with varying thresholds of stimulation at once suggests itself. But it would be premature to do more than call attention to this possibility, even though there is evidence that the chromatophores of other forms, particularly the melanophores, possess a double innervation.

Effects on Pigmentary Responses of Injecting Drugs into the Abdominal Cavity

To check the action of drugs upon the chromatophores in isolated scale preparations, observations were also made upon the results of injecting these same drugs into the abdominal cavity of the fish. On the whole the results were unsatisfactory and inconclusive, as the drugs used were only slightly effective if at all, except in lethal doses.

Nicotine

A strong solution of nicotine (1 drop in 1 cc.) produced violent convulsions immediately followed by a pronounced paling. This, however, lasted for only a few minutes, the animal reddening again with the

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approach of death, and by the time it was dead it was completely red. Hewer (1927) reports nicotine as first producing paling and then darkening when injected into the dab; all the chromatophores, lipophores as well as melanophores, reacting the same.

Physostigmine

One cc. of a 0.06 per cent to 0.013 per cent solution had no effect. With 2 cc. of 0.05 per cent to 0.065 per cent a reddening was observed. In such cases the animal usually died, always in a very red condition. Abolin (1926) found physostigmine to have no effect on the erythrophores of *Phoxinus*.

Pilocarpine

A 0.065 per cent to 0.15 per cent solution in 1 cc. doses produced some reddening, but not until one to two hours after the injection. Abolin (1926) observed no change in erythrophores of *Phoxinus* after pilocarpine injection.

Atropine

One cc. to 0.3 cc. of a 0.5 per cent to 0.05 per cent solution had no observable effect upon the color of the animal either on white or black backgrounds. However, with doses of 1 cc. of 1 per cent solutions there was a pronounced reddening, which in one case persisted for at least two hours. Osterhage (1932) observed in *Gasterosteus aculeatus* and *Rhodeus amarus* that the injection of atropine produced a pronounced reddening of the fins of the fish during the spawning season (May and June), but at other times of the year it only served to darken the animal. This was due presumably to the scarcity of erythrophores in the skin at times other than during the spawning season. Abolin (1926) reported atropine as being without effect on *Phoxinus* erythrophores.

Acetyl-choline

One cc. of 0.15 per cent solutions had no effect. Wunder (1931) observed in *Gasterosteus aculeatus* and *Rhodeus amarus* a slight reddening of the fins and belly following injection of acetyl-choline during the spawning season. Parker (1931) reports acetyl-choline as having a slight expanding effect on the chromatophores of *Fundulus*.

Cocaine

One cc. to 0.5 cc. of a 0.1 per cent to 0.25 per cent solution had no pronounced effects. In one case there was a slight paling, the meaning of which was doubtful. In another instance with 1 cc. of 0.25 per cent the animal died, becoming slightly redder just before death.

Adrenaline

A solution of 1/2,000 produced (0.6 cc.) pronounced paling in about twenty minutes. Abolin (1925) reports adrenaline as having no effect on *Phoxinus* xanthophores and Giersberg (1930) also failed to observe any effect of adrenaline upon the erythrophores of this form, although he did see a contraction of the erythrophores of *Macropodus,* an Asiatic fresh-water form.

Hypophysis Extracts

Extracts of the posterior pituitary of fishes were made by removing the gland and macerating it in distilled water. This extract was filtered and the filtrate injected into *Scorpæna.* Extracts made from the hypophyses of the tuna fish, *Thynnus thynnus,* and from *Trigla* sp., on injection into *Scorpæna ustulata* produced a paling which lasted for several hours. The tuna extract was made by macerating one gland in 5 cc. of water and the *Trigla* extract consisted of one gland in 1 to 2 cc. of water. Attempts were made to prepare an extract from the hypophyses of *Scorpæna ustulata* which would produce a contraction of the erythrophores in the same species, but none were successful. The failure to get a potent extract probably followed a failure to use a sufficient number of glands in preparing the solution. But lack of material prevented the preparation of extracts stronger than the ones used (four hypophyses in 2 cc. of water). This makes it doubtful whether any hormone secreted by the *Scorpæna* hypophysis is responsible for any of the observed pigmentary changes.

In *Phoxinus,* Abolin (1925) and Giersberg (1930) report infundin as producing melanophore and lipophore expansion. One of us (D. C. S.) had occasion some time ago to try the effect of an extract of trout pituitary (posterior lobe) on *Phoxinus,* and found it to produce a pronounced contraction of the melanophores and an equally pronounced expansion of the erythrophores. Hewer (1925) had previously observed extracts of cod pituitary to contract the melanophores of *Phoxinus* and to expand the xanthophores and erythrophores, and Matthews (1933) found extracts of *Fundulus* pituitary to contract the melanophores and expand the xanthophores of isolated *Fundulus* scales. However, both Hewer and Matthews doubt whether any hypophysial hormone is responsible for the color changes observed in the forms on which they worked, and we have seen that this is also probably true in the case of *Scorpæna.* Giersberg (1932), however, observed extirpation of the hypophysis in *Phoxinus* to abolish all color changes produced by the lipophores. Wunder (1931) and Osterhage (1932) saw an expansion of the erythrophores in the stickleback and bitterling following injection of commercial hypophysis extracts. Meyer (1931) observed contraction of the melanophores and expansion of the erythrophore in *Gobius* and *Pleuronectes* after hypophysis injection. In *Phoxinus, Fundulus, Gasterosteus, Rhodeus, Gobius,* and *Pleuronectes,* hypophysis extracts produce an expansion of the erythrophores or xanthophores, but in *Scorpæna,* fresh fish hypophyses extracts produce a contraction of the erythrophores. In those forms where expansion is the rule the erythrophores or xanthophores are relatively less active in comparison to the melanophores, and in the first four mentioned they are primarily associated with the production of nuptial coloration, except perhaps in *Fundulus,* and even in this form the xanthophores are probably most active during the spawning season. In *Scorpæna,* however, the erythrophores are the dominating pigment cells, the melanophores being subordinate in number and effect, and it is primarily the erythrophores which are responsible for the color changes the animal displays. It would seem as if in *Scorpæna* the erythrophores have assumed a different type of pigmentary activity than in the other forms mentioned.

It is perhaps well to remark here that in *Scorpæna* no qualitative difference between the responses of the erythrophores and the melanophores was ever seen. Within the same scale the erythrophores often reacted more rapidly than the melanophores, but the direction of the response was always the same. This again is in marked contrast to the condition found in some other forms where the melanophores and the erythrophores differ widely in the nature of their reactions. The xanthophores, however, are sharply differentiated from the erythrophores, particularly in respect to a greater sluggishness of behavior and a failure to pulsate in NaCl after $BaCl_2$.

SUMMARY

1. Evidence is presented to show that the erythrophores of *Scorpæna* will respond to nervous stimulation.

2. Isolated scale erythrophores of *Scorpæna* will show rhythmical pulsations in N/10 NaCl after treatment with N/10 $BaCl_2$.

3. Isolated scale erythrophores of *Scorpæna* will show the following responses when immersed in the following drugs:

a. Nicotine in weak solutions (1/100,000–1/10,000) produces a primary expansion followed by contraction. Moderately strong solu-

tions (1/10,000–1/2,000) produce a slight expansion followed by contraction. Strong solutions (1/2,000–1/100) produce a permanent expansion.

b. Physostigmine (0.0065–0.013 per cent) produces a " ringed " expansion.

c. Pilocarpine (0.016–0.04 per cent) produces a " ringed " expansion.

d. Atropine (0.025–0.0065 per cent) produces a " ringed " expansion. After atropine, however, the usual lasting contraction produced by N/10 KCl persists only a minute or so and is replaced by a permanent expansion. There is no change in the contracting effect of adrenaline after atropine.

e. Cocaine (0.1–0.01 per cent) produces a contraction.

f. Alcohol in strong solutions (10–5 per cent) produces first an expansion then a contraction and finally a " ringed " expansion. Weak solutions (1 per cent) produce a persistent contraction.

g. Acetyl-choline (0.1–0.01 per cent) had no effect.

4. No relation was found between the effect of drugs upon isolated scale erythrophores and their effects upon the color of the fish after injection into the abdominal cavity.

5. Hypophysis extracts of the tuna and *Trigla* produce a pronounced and lasting paling (erythrophore contraction) when injected into the abdominal cavity of *Scorpæna ustulata*.

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THE PENETRATION OF THE BLUE AND RED COMPONENTS OF DAYLIGHT INTO ATLANTIC COASTAL WATERS AND ITS RELATION TO PHYTOPLANKTON METABOLISM

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The following observations were undertaken as a continuation of the investigation of the conditions of submarine irradiation in relation to the biology of marine organisms. The measurements reported here and previously (Clarke, 1933) on the penetration of daylight into Atlantic waters serve as a basis for a comparison of this region with others which are being investigated intensively (Atkins and Poole, 1933; Utterback, 1933; Utterback and Boyle, 1933; Richardson, 1933; Erikson, 1933; Pettersson, 1934; and Pettersson and Landberg, 1934). The present measurements were made at two stations in the Gulf of Maine, one in the deep central part and one on Georges Bank, and at four localities in the region of Woods Hole. At the stations in the Gulf of Maine the vertical distribution of the phytoplankton was investigated (Braarud, 1934), complete hydrographic observations were made, and the diurnal migrations of copepods during the entire periods were studied (Clarke, 1934). Detailed information on the irradiation present at various depths is valuable not only in its immediate application to these studies of the plankton but also as an essential part of an intensive study of the oceanography of the Gulf of Maine (*cf.* Rakestraw, Gran, and Waksman, 1933). The observations in the neighborhood of Woods Hole were made in Woods Hole Harbor, Buzzards Bay, Vineyard Sound, and off Gay Head—localities chosen as representing four typical environments in this region. It is hoped that these measurements of light penetration will be useful in relation to certain of the biological problems being studied at Woods Hole, particularly those dealing with ecological relationships (*cf.* Allee, 1934). Some measurements of the photosynthesis of diatoms in this region are reported at the end of this paper.

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[1] Contribution No. 45.

APPARATUS AND METHODS

The photoelectric method previously described (Gall and Atkins, 1931; Clarke, 1933) was used. For the measurement of the blue component of daylight (3460Å–5260Å) the same deck photometer was employed. For submarine measurements the gas-filled potassium (sensitized) photoelectric cell was used in the water-tight case equipped with the four-conductor cable and the internal shutter. This combination is designated in the tables as Photometer 1*a*. The high sensitivity of the gas-filled cell made it useful for measurements at the deeper levels, but more trouble with changes of emission—especially near the surface—was experienced than with the vacuum cell. The cell was always glowed before each reading and all the other precautions previously described were taken in these observations.

For measurements in the red region of the spectrum two Type PJ–14 vacuum cæsium on cæsium oxide cathode photocells were used (kindly furnished by the General Electric Co.) which have peaks of maximum sensitivity in the near ultra-violet and red regions of the spectrum (Fig. 1). One of these cells was mounted in a newly constructed deck photometer and the other in sea photometer case No. 2—this latter combination being designated in the tables as Photometer No. 2*b*. To limit the radiation reaching the cells, each was mounted under a Schott-Jena Type RG–1 glass filter, 5 cm. square and 2 mm. thick, which cut out all radiations shorter than 6000Å, and a water-cell 2 cm. in depth and containing a 1.5 per cent solution of $CuCl_2$ which absorbed radiations of wavelength longer than 7000Å (*cf.* Coblentz, 1911). The filters were placed against the inside surface of the photometer window, the spaces between the filters and between the upper filter and the window being filled with Canada balsam to minimize reflection loss at the glass faces. When examined spectroscopically the duplex filters were found to have a sharp cut-off at 6000Å and a graded cut-off at 7000 to 7100Å with approximately 3 per cent transmission at 7000Å.

The spectral sensitivities of the cæsium photocells without the diffusing disc and filters were determined by the General Electric Research Laboratory under the direction of Dr. L. R. Koller (Fig. 1). The photocells mounted in the photometer cases under the filters were standardized for the spectral range 6000Å–7000Å by the same method as that employed for the blue-sensitive photometers (Clarke, 1933).

No changes in emission due to "photoelectric fatigue" were observed in moderate steady light in using the cæsium cells, but after continued exposure to bright sunlight on the deck of the ship decreases in the sensitivity of these cells were found amounting to 14 per cent. The presence of "dark currents" in the deck photometer required frequent readings of the response with the photometer shutter closed during a series of observations. All measurements below the surface have been corrected for "external reflection" and "internal reflection" by multiplying by the factor 1.09 as before.

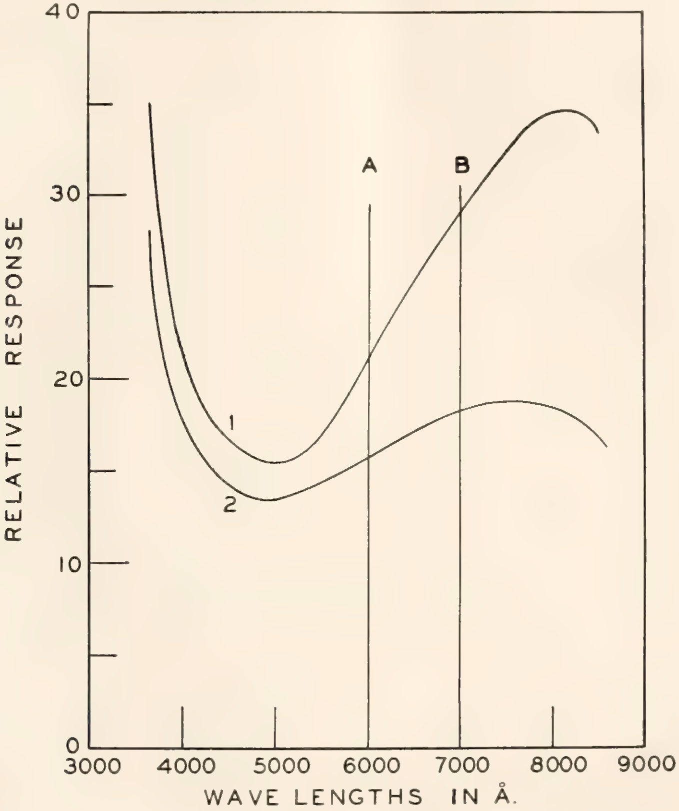

Fig. 1. Comparison of spectral sensitivities of red-sensitive photocells. (General Electric Research Laboratory.) Curve 1. No. 31,613. Cæsium on cæsium oxide—sea cell. Curve 2. No. 31,797. Cæsium on cæsium oxide—deck cell.

Each curve represents the relative response to an equal energy spectrum of the photocell alone.

In practice the cells are considered to be sensitive to the range between the vertical lines *A* and *B* as limited by the red RG–1 filter and the $CuCl_2$ filter cell.

Observations

The observations made on the penetration of blue and red light are summarized in Tables I and II. Curves showing the percentage of the

TABLE I

Summary of Observations in the Gulf of Maine

	Blue (3460Å–5260Å)			Red (6000Å–7000Å)			
Series No.	201	202	203	204	205	206	207
Station No.	1722	1722	1723	1722	1722	1723	1723
Lat. N.	42°50′	42°50′	41°19′	42°50′	42°50′	41°19′	41°24′30″
Long. W.	67°05′	67°05′	66°40′	67°05′	67°05′	66°40′	66°42′30″
Date, 1933	7/15	7/16	7/17	7/15	7/16	7/17	7/18
Local sun time (24-hr. scale)	1425–1515	1230–1440	1200–1410	1230–1340	1030–1200	1430–1535	1210–1325
Av. altitude of sun	48°	59°	63°	65°	64°	47°	56°
Photometer No.	1a	1a	1a	2b	2b	2b	2b
Av. transmissive exponent, *k*, over depth studied	0.11	0.11	0.14	0.31	0.32	0.35	0.34
Irrad. at depth of 1 meter (% surface light)	77	89	64	43	53	52	46
Depth at which irrad. is reduced to 1%, *meters*	40	34	26	12	12	11	12
Range of irrad. on deck during series *microwatts/cm.*^{2}	4,500–8,200	5,600–8,600	11,900–13,400	13,900–12,200	10,700–8,700	11,000–5,600	10,000–11,400
Sea (Douglas sea scale)	Slight	Smooth	Smooth heavy swell	Slight	Smooth	Smooth heavy swell	Slight heavy swell
Sky	Many clouds	Over-cast, fog	Clear, fog	Few clouds	Over-cast, fog	Clear, fog	Clear, fog

TABLE II

Summary of observations in the Woods Hole region. Woods Hole harbor, Series 122 and 211: bottom at 23 meters, tide flooding, *i.e.* water flowing in from Buzzards Bay. Off Gay Head, Series 208 and 212: bottom at 30 meters, current running west until 1537. Buzzards Bay, Series 209 and 213: bottom at 15 meters, high tide at New Bedford at 0920. Vineyard Sound (halfway between Nobska Point and West Chop), Series 210 and 214: bottom at 22 meters, current running east until 1500.

	Blue (3460Å–5260Å)				(Red (6000Å–7000Å)			
Series No.	122	208	209	210	211	212	213	214
Location	W. H. Harbor	Off Gay Head	Buzzards Bay	Vineyard Sound	W. H. Harbor	Off Gay Head	Buzzards Bay	Vineyard Sound
Lat. N.	41°31′	41°20′	41°33′	41°30′	41°31′	41°20′	41°33′	41°30′
Long. W.	70°40′	70°57′	70°42′25″	70°36′	70°40′	70°57′	70°42′25″	70°36′
Date	Aug. 22 1932	Sept. 2 1933	Sept. 6 1933	Sept. 8 1933	Aug. 30 1933	Sept. 2 1933	Sept. 6 1933	Sept. 8 1933
Local sun time (24-hr. scale)	1339–1429	1320–1445	1011–1210	1040–1145	1410–1540	1150–1305	1215–1350	1310–1450
Av. altitude of sun	50°	47°	54°	54°	40°	56°	53°	46°
Photometer No.	1	1a	1a	1a	2b	2b	2b	2b
Av. transmissive exponent, *k*, over depth studied	0.49	0.27	0.51	0.36	0.62	0.37	0.61	0.59
Irrad. at depth of 1 meter (% surface light)	48	79	50	47	29	39	43	42
Depth at which irrad. is reduced to 1%, *meters*	8	16	10	12	6	10	7	8
Range of irrad. on deck during series, *microwatts/cm.*^{2}	11,800–12,400	14,000–11,000	12,000–9,000	14,000–13,000	7,950–5,800	14,250–13,400	11,050·9,400	11,500–10,200
Sea (Douglas sea scale)	Calm	Smooth to slight	Smooth to slight	Smooth	Calm	Smooth to slight	Smooth	Slight
Sky	Clear	Clear	Clear	Clear	Clear	Clear	Clear	Clear

surface light reaching the various depths for all the series appear in Figs. 2 to 5. Considering first the penetration of blue light, we find that the average transparency at Station 1722 in the Gulf of Maine is slightly greater than that at Station 1053 (Clarke, 1933[2]). This is to be expected since Station 1722 is nearer the open ocean. The average transparency on Georges Bank (Station 1723), where the shallower water may be stirred down to the bottom, is about the same as at Stations 1285, 1286 and 1287 (previously reported) in deeper water but much nearer shore. The transparency of the water for blue light is from 2 to 4½

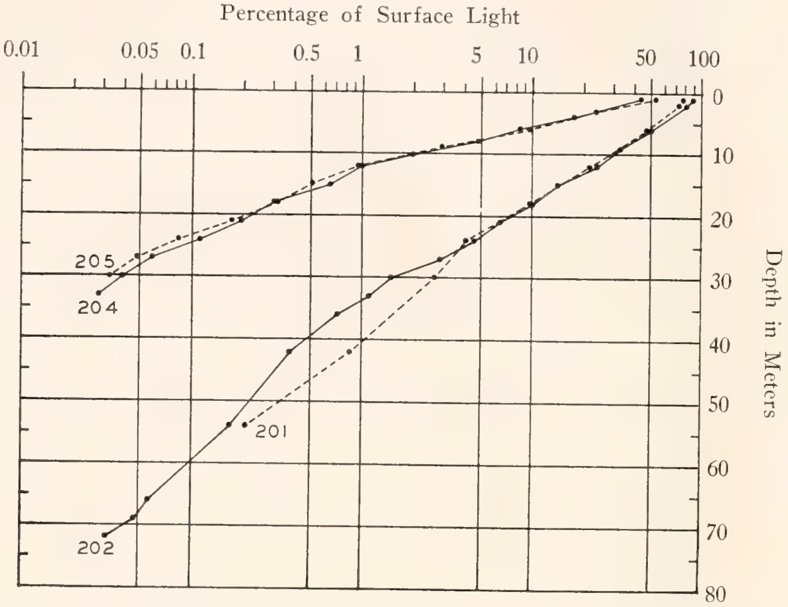

Fig. 2. Relation between depth and irradiation expressed as a percentage of the light just over the surface (logarithmic scale). Series number indicated at the end of each curve.

Station 1722: Gulf of Maine, deep basin.
Series 201 and 202—blue light.
Series 204 and 205—red light.

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[2] The following corrections should be made in "Average altitude of sun" in Tables II and III, Clarke, 1933 Biol. Bull., vol. 65, p. 317.

Table II

Series No.	1	2	3	4	5	6	7	8	9	10	11	12	13
Av. altitude of sun	58°	54°	58°	61°	62°	64°	65°	65°	62°	61°	24°	67°	54°

Table III

Series No.	13	112	113	114	115	116	117	118	119	120	121	122
Av. altitude of sun	54°	62°	1°	28°	37°	41°	68°	6°	22°	58°	57°	50°

times lower in the Woods Hole region than in the Gulf of Maine. The water of Buzzards Bay (Series 209) was found to have a slightly greater average transmissive exponent than the water of Woods Hole Harbor (Series 122). However, it is the turbidity of the water layer just over the bottom in Buzzards Bay which reduces the average transparency for the whole series, the upper strata being somewhat more transparent. In Woods Hole Harbor this situation is just reversed, the surface water being slightly less transparent than the deeper strata. The result is that daylight penetrates to a depth of 10 meters in Buzzards Bay before it is reduced to 1 per cent of its surface value whereas a corresponding reduction has taken place in the Harbor at 8 meters. The water off Gay Head (Series 208) is about half as clear as that characterizing the Gulf of Maine, and the transparency in Vineyard Sound (Series 210) is intermediate between that found off Gay Head and in Buzzards Bay. The Gay Head and Vineyard Sound regions correspond rather closely to the San Juan Archipelago in transparency to blue light (Utterback and Boyle, 1933).

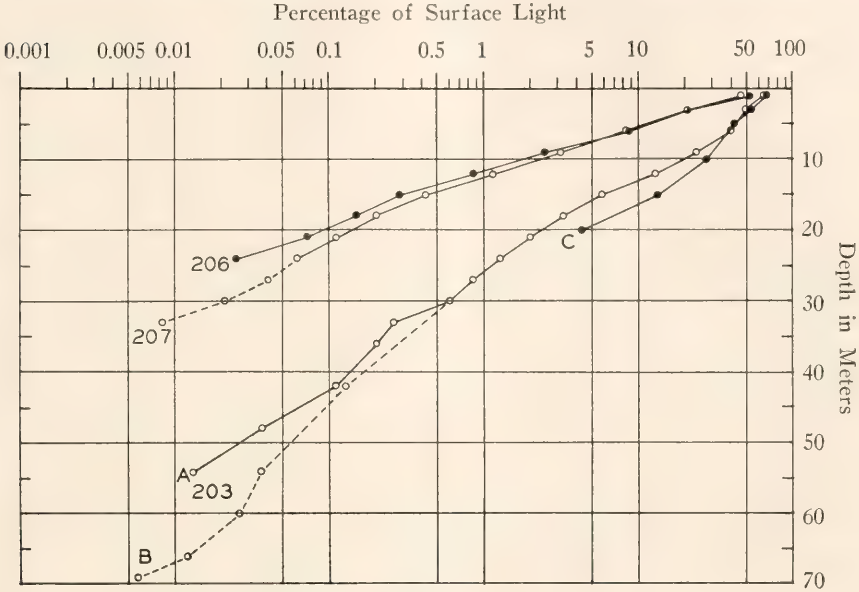

FIG. 3. Station 1723. Georges Bank.
Series 203—blue light.
a. Regular measurement.
b. Measurement of deeper strata with opal glass removed from photometer.
c. Measurement of upper strata repeated following day.
Series 206 and 207—red light. Broken portion of curve 207 represents measurement with opal glass removed.

The average transparency for red light in the deeper part of the Gulf of Maine (Station 1722) is slightly higher than that found by Atkins and Poole (1933) off Rame Head, England, although there is a closer agreement when only the range from 1 to 10 meters is used for comparison. On Georges Bank (Station 1723) the transparency for red light falls between that found off Rame Head and in Whitsand Bay, for the range 1 to 5 meters, and is lower than at Station 1722. The penetration of red light into the water off Gay Head (Series 212) is only slightly lower than on Georges Bank and is higher than would be expected for the locality. The waters of Vineyard Sound (Series 214), Buzzards Bay (Series 213), and Woods Hole Harbor (Series 211) are all very similar in transparency, but there is a slight increase in turbidity in the order mentioned. The transparency is approximately one-half that found at Station 1722.

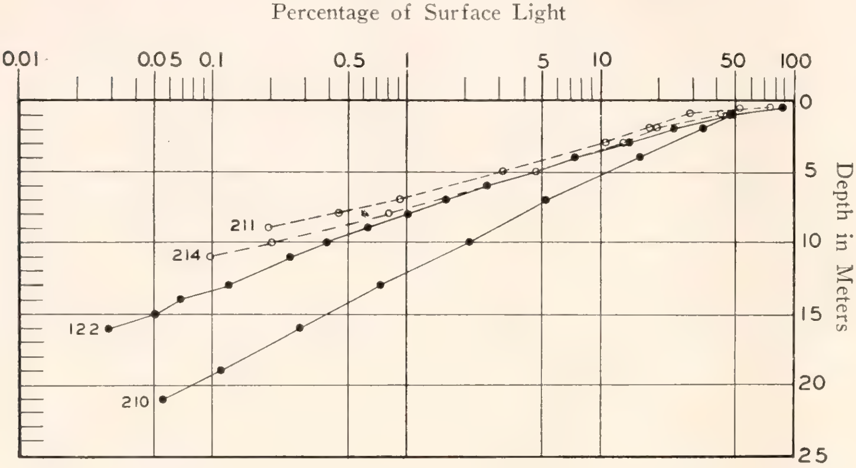

Fig. 4. Series 122. Blue light. Woods Hole Harbor.
Series 210. Blue light. Vineyard Sound.
Series 211. Red light. Woods Hole Harbor.
Series 214. Red light. Vineyard Sound.

In some of the series the transmissive exponent in the red decreases with depth. This is especially evident at Stations 1722 and 1723 and to a lesser extent in Series 212 where the depth of the water allows for some "layering." Atkins and Poole (1933) found similar results for certain observations in British waters, and Utterback and Boyle (1933) for measurements made in the waters of the San Juan Archipelago. Irradiation in the deeper layers is probably at the shorter wave-lengths so that the energy reaching the photocell through the Schott RG–1 filter would be at or near to 6000Å, *i.e.* in the orange.

A comparison of the energy in the blue and red regions of the sun's spectrum is given in Fig. 6 as measured on the deck of the ship over the period of daylight on July 16, 1933 at Station 1722. For the spectral limits measured the agreement in the amount of energy is close. Since the sky during the period covered was overcast with some fog, the red radiation was probably transmitted more readily than the blue due to more scattering at the shorter wave-lengths. Scattering of blue light may also help to explain the close agreement at sunset when the red component would normally have the higher value.

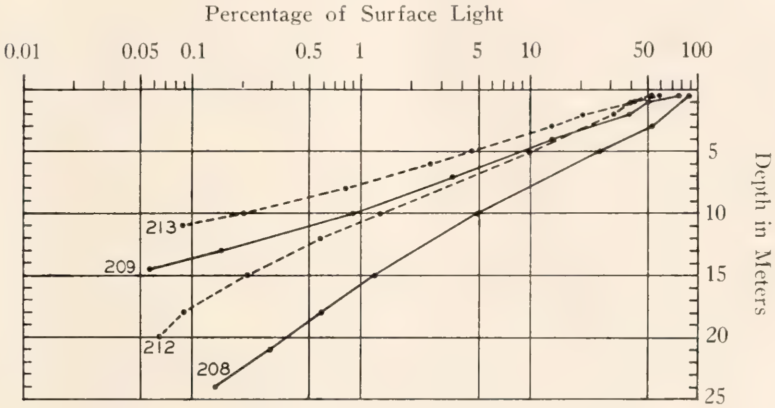

Fig. 5. Series 208. Blue light. Off Gay Head.
Series 209. Blue light. Buzzards Bay.
Series 212. Red light. Off Gay Head.
Series 213. Red light. Buzzards Bay.

DISCUSSION

The curves for the penetration of both blue and red light show marked differences in their degree of irregularity. Thus the curves for the Gulf of Maine are quite jagged in comparison with those for the Woods Hole region. This may be due in part to the much rougher condition of the sea experienced in the Gulf of Maine. It seems probable, however, that a more important factor is the thorough stirring caused by the very strong tidal currents of the Woods Hole region. Temperature measurements (such as those in Table III) show that the water is very nearly homogeneous from top to bottom. The irradiation measurements obtained in Vineyard Sound (*e.g.* Series 210), famous for its strong tide, fall on a remarkably straight line. Moreover,

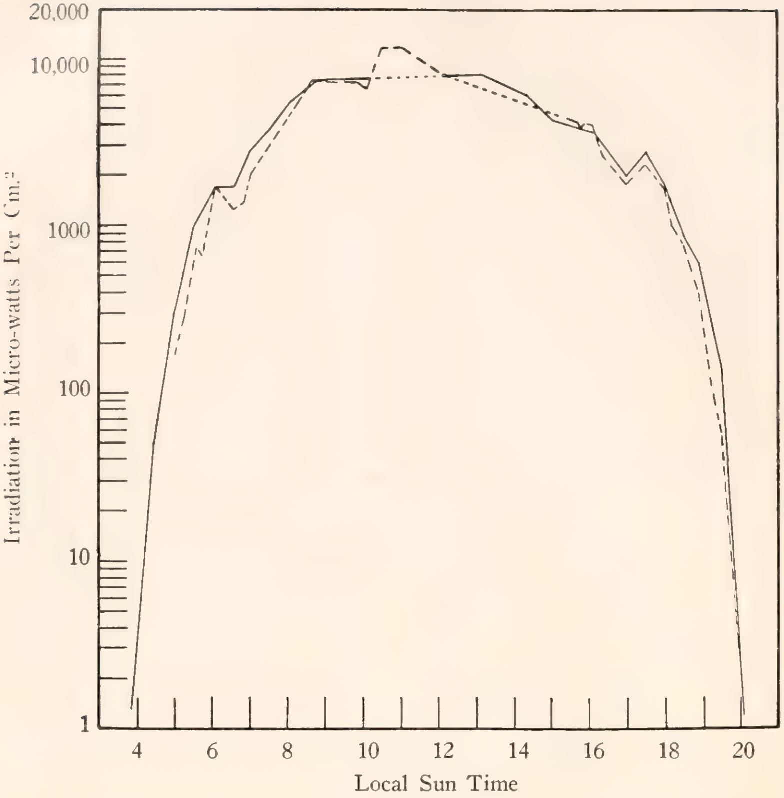

FIG. 6. Comparison of the blue and red components of daylight measured on deck during July 16, 1933 at Station 1722. Solid line indicates blue light, broken line indicates red light.

this regularity indicates that the method of measurement is highly reliable and that the irregularities in the values obtained at other localities are not to be assigned to fluctuation of the photometers.

If changes in the slopes of the curves really are caused by water layers of different transparency, it is difficult to understand why changes in the blue curve are not represented by corresponding changes in the red curve for the same locality, and vice versa, since the two sets of measurements were ordinarily taken within two hours of each other. Examples of such lack of conformity are seen, particularly, in the Gulf of Maine Series. The presence in different water layers of material having a highly selective effect on light would account for these irregularities, but the actual existence of such material has yet to be demonstrated. The differences between the three segments (*a*, *b*, and *c*) of Series 203 certainly indicate a great lack of homogeneity in this case.

The relative clearness of the bodies of water considered is the same whether arranged in order of average transparency to blue light or to red light except for the Woods Hole Harbor and Buzzards Bay series. The transmissive exponent for blue light is slightly higher in Buzzards Bay than in Woods Hole Harbor, whereas the reverse is true for red light. However, the transparency of the harbor water is very close to that of the Bay water in both cases, and for blue light at least it is distinctly different from that of Vineyard Sound. This is in agreement with the fact that when the tide is flowing into the harbor, the water is derived chiefly from Buzzards Bay (*cf.* Fish, 1925).

Red light is absorbed faster than blue light in all of these bodies of water, but it is significant to note that the ratio of the transmissive exponents is not constant. The ratios of the exponents for red light to the exponents for blue light follow:

Station 1722	2.9
Station 1723	2.5
Off Gay Head	1.4
Vineyard Sound	1.6
Woods Hole Harbor	1.3
Buzzards Bay	1.2

It is seen that, in general, as the water becomes more turbid the difference between the penetration of blue and red light becomes less. This would seem explicable on the basis of the greater scattering effect of increasing amounts of suspended particles on radiation of short wave-length than on radiation of long wave-length. The ratio of the exponents for red and blue light in the Gulf of Maine is about the same as in Whitsand Bay (Atkins and Poole, 1933). The ratio in the Woods Hole region is similar to that in the San Juan Archipelago (Utterback and Boyle, 1933).

Note on the Metabolism of the Phytoplankton

Of previous investigations on the photosynthesis of aquatic plants in relation to the spectral distribution of the irradiation (*cf.* Klugh, 1930), few deal specifically with the marine phytoplankton. It is therefore difficult to correlate the present measurements of the penetration of light with the vertical distribution and metabolism of diatoms, etc. However, since photosynthesis is of primary significance in the economy of the sea, it seems important to report such observations as are available. Laboratory experiments indicate that intensity is more important than color for the photosynthesis of diatoms, but since chromatic adaptation occurs, it is necessary to know the spectral composition as well as the total intensity of the irradiation present (Stanbury, 1931). Klugh (1930) concludes that certain pigments present in the cell may act as photo-sensitizers for the chlorophyll in plants growing at depths lower than the first few meters. Schreiber (1927) exposed various species of diatoms and dino-flagellates to general radiations and showed that the optimum radiant energy for growth differed widely in different forms.

On August 16 and August 22, 1932, Dr. H. H. Gran carried out measurements of the metabolism of diatoms in Woods Hole Harbor at the same time that the observations on the penetration of blue light were being made (Series 120, 121, and 122). Comparable values for red light may be obtained from Series 211. For the experiments sea water with the contained diatoms was taken from Vineyard Sound and placed in glass-stoppered bottles of 200 cc. capacity. The bottles were placed horizontally in shallow wire baskets and these were lashed at 3-meter intervals to a weighted rope hung from the south side of the anchored boat. Each basket contained two bottles fully exposed to light, and four of the baskets contained in addition one bottle which was completely covered with black cloth, thus providing an opportunity to measure respiration in the absence of photosynthesis. The oxygen present in two control bottles was measured, using the Winkler method at the beginning of the exposure period, and similar measurements were made on all the other bottles at the end. The bottles were exposed at their respective depths for about 4 hours during the middle of the day.

The experiments were not intended to yield more than approximations and the results are somewhat irregular. However, the location of the depth at which the oxygen consumed in respiration is just balanced by the oxygen evolved in photosynthesis (*i.e.* the " compensation point ") seems quite definite. The results of the experiment on August 22 are presented in Table III, where it is seen that the bottles at the 3-meter and 6-meter levels showed an increase in oxygen whereas those at greater depths showed a decrease. Since on August 16 the shallowest bottle was placed at 8 meters and all the bottles showed a decrease of oxygen content at the end of the experiment, we may place the " compensation point " for Woods Hole Harbor at about 7 meters. At this depth the average irradiation present during the experiment was as follows:

Blue	192 microwatts/cm.^{2}
Red	108 microwatts/cm.^{2}

On June 1, 1934, Dr. A. C. Redfield carried out similar measurements, using the same methods, while the " Atlantis " was anchored at

Station 2212 in the Gulf of Maine 40 miles east of Cape Cod. A diatom haul made on Georges Bank was allowed to stand for 2 days and the suspended material decanted off and diluted. Bottles containing this diatom suspension were placed at 10-meter intervals down to 50 meters and left for 7 hours. When the tide was running strongly the bottles were brought slightly nearer the surface, as is indicated in Table IV. The results are satisfactorily consistent and show that photosynthesis took place down to at least 40 meters. The progressive diminution of oxygen produced from the surface downwards is a result both of the decreased irradiation and of the lowered temperature. The amount of oxygen consumed in respiration likewise diminished with depth and is correlated with the temperature. The "compensation point" occurred at 30–24 meters.

TABLE III

Metabolism of diatoms in Woods Hole Harbor. Exposure period: 1002–1427, August 22, 1932. Range of irradiation on deck during exposure period: blue (3460Å–5260Å): 12,400–11,600 microwatts/cm.^{2}; red (6000Å–7000Å): 12,000–11,200 microwatts/cm.^{2}* Oxygen present before exposure: 6.74 cc./liter. Oxygen present in covered bottles at end of period: 6.64 cc./liter.

Depth	Temp.	Average irradiation blue	Average irradiation red	Oxygen after exposure	Change in oxygen
meters	° *C.*	*microwatts/cm.*^{2}	*microwatts/cm.*^{2}	*cc./l.*	*cc./l.*
3	21.40	1700	1240	7.06	+0.34
6	21.39	370	197	6.81	+0.07
9	21.33	98	23	6.72	−0.02
12	21.27	27	3	6.71	−0.03
15	21.26	7.2	—	6.73	−0.01
18	21.27	—	—	6.67	−0.07

*This value assumed from information obtained a year later on the ratio of the blue and red components of daylight.

Since no measurements of irradiation were made during these observations, it is impossible to make an exact comparison of the results with those obtained in Woods Hole Harbor. However, it is worth pointing out that in the Gulf of Maine the water is roughly three times as transparent as in Woods Hole Harbor and the "compensation point" lies about three times as deep. Marshall and Orr (1928) found the "compensation point" in Loch Striven to occur between 20 and 30 meters, but a satisfactory comparison with their observations is similarly difficult because of the different methods used. The calculation of light intensity for Loch Striven made by Atkins and Poole (1933) cannot readily be compared with ours since the values arrived at are for irradiation over the whole spectrum throughout the day and ours are concerned with two limited regions of the spectrum for a few hours. It appears, however, that the water of Loch Striven, like the water in the Gulf of Maine, is somewhat more than three times as transparent as the water in Woods Hole Harbor and the "compensation point" for the whole day lies correspondingly deeper.

Table IV

Metabolism of diatoms in the Gulf of Maine. Exposure period: 0444–1354, June 1, 1934. Sky: cloudy, becoming clear and later overcast. Sea: smooth, moderate swell.
Oxygen present before exposure: 7.80
7.83
7.83
av. 7.82 cc. per liter.

Depth	Temp.	Condition of bottles	Oxygen after exposure	Change in oxygen	Oxygen produced
meters	° *C.*		*cc./l.*	*cc./l.*	*cc./l.*
0	10.75	covered	7.11	−0.71	
		exposed	9.42	+1.60	+2.31
		"	9.44	+1.62	+2.33
10–8	10.60	covered	7.32	−0.50	
		exposed	8.40	+0.58	+1.08
		"	8.44	+0.62	+1.12
20–16	10.0	covered	7.42	−0.40	
		exposed	8.07	+0.25	+0.65
		"	8.02	+0.20	+0.60
30-24	6–9	covered	7.61	−0.21	
		exposed	7.81	−0.01	+0.20
		"	7.72	−0.05	+0.16
40–32	5–6	covered	7.54	−0.28	
		exposed	7.66	−0.16	+0.12
		"	7.71	−0.11	+0.17
50–40	4–5	covered	7.61	−0.21	
		exposed	7.59	−0.22	+0.01
		"	7.64	−0.18	−0.03

From the samples of phytoplankton collected in July, 1933 at Stations 1722 and 1723 and subsequently subjected to a quantitative study by Mr. T. Braarud (1934) it was found that the dominant form as to numbers at both stations was the coccolithophoride *Pontosphæra Huxleyi,* which reached its maximum at approximately 18 meters at Station 1722 and at near 14 meters at Station 1723. Braarud (1934) states that the production of *Pontosphaera* seems to have been confined to the layer in which the temperature was higher than 10° C. At these depths where the maximum of *Pontosphæra* occurred the blue light was reduced to 9.9 per cent and 11.4 per cent respectively and the red light to 0.31 per cent and 0.50 per cent respectively. Taking 13,000 and 11,000 microwatts/cm.^{2} for approximate values of blue and red irradiation at the surface at noon on a clear day, we obtain light intensities of 1290 and 1480 μW/cm.^{2} for the blue and of 34 and 55 μW/cm.^{2} for the red for these depths. It will be noted that the blue irradiation is greater and the red irradiation less than that found at the "compensation point" in Woods Hole Harbor. However, no conclusions can be drawn from these facts as yet since the effect of the different parts of the spectrum on photosynthesis and the influence of other environmental factors are so poorly understood.

It is of interest to inquire whether the phytoplankton population has an appreciable effect on the reduction of light with depth (*cf.* Atkins and Poole, 1933). No sharp changes in the slopes of the curves occur at the levels of maximum abundance of *Pontosphæra*. The diatom, *Guinardia flaccida* similarly has no noticeable influence upon the transparency. This species, being about fifty times larger than *Pontosphæra,* is the dominant form in respect to bulk at Station 1723 and occurs in maximum numbers at 30 meters.

It is evident that the whole question of the relationship between submarine irradiation and the metabolism of the phytoplankton must be much more thoroughly investigated both in the laboratory and at sea. In particular, for an understanding of the biology of Atlantic coastal waters we lack measurements of the penetration of the green and yellow parts of the spectrum. We now have a general idea of the vertical distribution of the plankton in these waters and of the conditions of submarine irradiation in respect to blue and red light.

SUMMARY

1. Photoelectric measurements of blue and red submarine irradiation were made at two stations in the Gulf of Maine and at four localities in the Woods Hole region. The same apparatus was used as before (Clarke, 1933) with the addition of two cæsium on cæsium oxide photocells mounted under Schott-Jena RG–1 filters and $CuCl_2$ water-cells which limited the radiation received to 6000Å to 7000Å.

2. The bodies of water considered may be arranged in order of decreasing transparency to both blue and red light as follows: (*a*) the

deep basin of the Gulf of Maine, (*b*) Georges Bank, (*c*) Off Gay Head, (*d*) Vineyard Sound, (*e*) Buzzards Bay and Woods Hole Harbor. The ratios of the transmissive exponents for red light to those for blue light in each locality vary from 2.9 to 1.2, the higher values being characteristic of clearer water.

3. These measurements are discussed in relation to the local hydrographic conditions, and are compared with observations made in the Plymouth region and in the San Juan Archipelago.

4. Measurements made by Dr. H. H. Gran on the metabolism of diatoms in bottles suspended at different depths in Woods Hole Harbor indicated that the " compensation point " was located at a depth of about 7 meters. Similar measurements carried out by Dr. A. C. Redfield gave a depth of 24–30 meters for the " compensation point " in the Gulf of Maine.

5. Observations reported by Mr. T. Braarud (1934) on the vertical distribution of the phytoplankton at the same stations in the Gulf of Maine are discussed in relation to the penetration of light.

The authors desire to express their thanks to Mr. R. T. Montgomery for his kind assistance in the measurements made at sea.

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A NOTE ON THE PHYTOPLANKTON OF THE GULF OF MAINE IN THE SUMMER OF 1933

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(*From the Woods Hole Oceanographic Institution*,[1] *Woods Hole, Mass.*)

The rather scanty quantitative data on the phytoplankton distribution in the Gulf of Maine in summer are mostly from the western part, collected during 1931–32 for the International Passamaquoddy Fisheries Commission (Gran and Braarud, 1934). In addition, Gran (1933) studied the phytoplankton distribution in the summer of 1932, from material collected at several stations. The phytoplankton described in this paper was obtained from water bottle samples taken by Clarke at two stations in the Gulf of Maine during his work on the light conditions in the water and on the vertical migration of the zoöplankton. These stations represent much the same part of the Gulf as do two of the stations from which the 1932 material was collected. The 1932 observations were made on August 3–4, while those in 1933 occurred about two weeks earlier in the season, on July sixteenth and eighteenth. The present collections supplement our knowledge of the distribution of phytoplankton in this region and also provide data for the study of the relationships between the plant population and the penetration of daylight into the water. Since the latter aspect of the work is discussed by Clarke and Oster (1934) for these stations, I shall confine myself to a short account of the distribution of the phytoplankton. The question of the importance of diatoms in light absorption has been discussed by Poole and Atkins (1929) and by Marshall and Orr (1930).

The surface layers at Station 1722 (in the eastern part of the Gulf of Maine) consisted of presumably local water of low salinity (32.07 to 32.19°/₀₀), while at Station 1723 the uppermost layer was of Atlantic origin, having a salinity between 33.12 and 33.54°/₀₀. (See Table III.) In spite of this conspicuous difference in the characteristics of the water, the phytoplankton communities at the two stations had much the same character. The form numerically dominant at both localities was the coccolithophoride *Pontosphæra Huxleyi*, which was abundant at a depth of 1 to 25 or 30 meters. At both stations the maximum number was found at an intermediate depth (15, 18 meters), and the production of *Pontosphæra* seems to have been confined to the layer in which the temperature was more than 10° C. This is corroborated by the oxygen distribution, as the layer in which there was supersaturation of oxygen is the same as that in which the numbers of *Pontosphæra* were high. The only exception is Station 1723, where at 25 meters 30,000 cells of *Pontosphæra* per liter were found and yet there was no supersaturation in oxygen. Here the temperature was only 8.67° C. The general distribution of *Pontosphæra Huxleyi* seems clearly to indicate that it is a moderately thermophile form which reproduces very slowly at temperatures lower than 7° C. (Gran, 1930, p. 32). It seems probable, therefore, that this population at 25 meters was the result mainly of the sinking of the abundant population of *Pontosphæra* from the layers above (see Table III), and that the local production of *Pontosphæra* at this depth was small on account of the low temperature and the poor light conditions.

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[1] Contribution No. 46.

At Station 1723 (on Georges Bank) diatoms were fairly abundant, but only two species were recorded in any great numbers. In the upper warm layer *Detonula confervacea* occurred in small quantities in every sample, with a maximum number of 1,300 cells per liter at 15 meters—the same depth at which the maximum number of *Pontosphæra* occurred. *Guinardia flaccida* was distributed mainly below 20 meters, with a maximum of 5,300 cells per liter at 30 meters. This species has previously been recorded as a form which commonly, if not always, has occurred in the waters over the Bank (Bigelow, 1926; Gran, 1933). The *Ceratia* must have been very scarce at Station 1723, since in the centrifuged samples only a few specimens of *Ceratium longipes* and *C. tripos* were recorded. A colorless flagellate (*Bodo marina?*) was common at all depths.

At Station 1722 very few diatoms were observed, and no forms except *Pontosphæra Huxleyi* occurred in great numbers. The *Ceratia* were as scarce as at Station 1723. The *Guinardia* population was absent here, or at least present in numbers too small to be recorded in the volume of water which was centrifuged. Except for the *Guinardia,* the communities were very much the same in the two localities, but quantitatively poorer at Station 1722.

Although Gran's observations (1933) were not made in exactly the same localities as the present ones, an approximate comparison may be made between the summer vegetation at Georges Bank and in the eastern part of the Gulf of Maine in the years 1932 and 1933. The difference in time of season, viz., two weeks, is presumably of no great importance, since the conditions do not seem to change quickly at this time of the year. In the Gulf proper, a remarkable difference between the two years is found in the quality of the phytoplankton. In 1932 di-

TABLE I

Station 1722, July 16, 1933. Latitude, 42°50′ N, Longitude, 67°05′ W.

Depth in meters	1	6	12	18	30	42	54	66	78
Temperature, ° C	13.17	13.04	12.52	11.39	10.46	8.50	7.01	6.59	5.77
Salinity, ‰	32.07	32.07	32.09	32.11	32.19	32.42	32.63	32.75	32.80
Oxygen, cc. per liter	6.48	6.41	6.65	6.87	6.85	6.60	6.48	6.42	6.20
Oxygen, % saturation	107	105	108	109	107	99	94	92	88
PO_4', mg. per cu.m.	29	29	29	29	30	55	60	66	78
NO_3', mg. per cu.m.	11	13	9	8	11	55	80	85	104
Number cc. examined	10	10	10	10(50)	10(50)	10	10	50	50
Phytoplankton, cells per liter:									
Chætoceros sp.	—	—	—	—	—	—	—	20	—
Corethron hystrix	—	—	—	—	—	—	—	20	—
Detonula confervacea	200	100	—	200	100	—	200	60	—
Coccolithus pelagicus	—	—	—	—	—	—	—	—	40
Pontosphæra Huxleyi	146,000	105,400	198,000	308,000	130,200	13,200	9500	6460	1880
Eutreptia viridus	—	—	—	200	—	—	—	—	—
Ceratium longipes	100	—	—	—	—	—	—	—	—
Exuviælla baltica	—	400	—	100	—	—	—	—	—
Gymnodinium Lohmanni	—	—	200	200	200	—	100	80	—
" sp.	600	400	700	300	100	—	100	160	200
Minuscula bipes	—	—	—	100	—	—	—	20	—
Oxytoxum gracile	—	—	100	—	—	—	—	—	—
Peridinium sp.	—	200	—	—	—	—	—	20	—
Acanthostomella norvegica	—	—	—	—	200	—	—	20	—
Laboea conica	—	—	—	100	—	—	—	20	—
" emergens	—	100	—	—	100	—	—	20	—
" reticulata	—	—	500	600	100	—	—	—	—
" strobila	—	—	—	100	—	—	—	—	—
Lohmanniella oviformis	200	300	—	800	2300	100	—	40	—
" spiralis	—	—	300	200	—	—	—	120	60
Mesodinium rubrum	—	—	100	—	—	—	—	—	—
Bodo marina?	6700	3800	9500	5300	5500	300	500	80	20
Forms not identified	1100	300	900	1900	3800	100	300	220	100

TABLE II

Station 1723, July 18, 1933. Latitude, 41°19′ N, Longitude, 66°40′ W.

Depth in meters	1	5	10	15	25	30	40	50	60
Temperature, ° C	15.45	15.47	15.39	13.92	8.67	8.47	8.44	8.21	8.07
Salinity, $^0/_{00}$	33.12	33.15	33.54	33.27	32.51	32.53	32.56	32.56	32.60
Oxygen, cc. per liter	6.18	6.44	6.30	6.58	6.40	6.38	6.24	6.23	6.33
Oxygen, % saturation	107	112	110	111	97	96	94	93	94
PO_4', mg. per cu.m	28	31	28	49	65	68	76	72	75
NO_3, mg. per cu.m	14	11	8	9	53	54	67	62	62
Number cc. examined	25	10	10	10	10(25)	10	10	10	10
Phytoplankton, cells per liter:									
Chætoceros decipiens	—	—	—	—	—	900	500	—	—
Corethron hystrix	40	—	100	—	—	—	—	—	—
Coscinosira œstrupi	—	—	—	—	—	100	—	100	500
Detonula confervacea	400	600	600	1300	200	—	—	100	—
Guinardia flaccida	—	100	—	700	1800	5300	3700	3900	2200
Leptocylindrus danicus	—	—	—	200	800	—	—	—	—
Melosira sp.	—	—	—	—	—	100	—	—	—
" sulcata	—	—	—	—	—	—	400	—	—
Nitzschia closterium	40	—	—	—	—	—	100	100	100
" delicatissima	320	—	500	100	200	—	—	400	300
" seriata	—	—	—	—	—	200	—	—	—
Rhizosolenia alata	120	300	—	—	—	—	—	—	—
" fragilissima	40	—	—	—	—	100	—	—	—
" semispina	—	—	100	—	—	—	—	—	—
" styliformis	—	—	—	350	200	1500	—	800	50
Thalassiosira decipiens	—	—	—	—	—	200	—	—	—
" gravida	—	—	—	—	—	—	—	—	100
" sp.	—	—	200	—	—	—	—	—	—
Coccolithus pelagicus	—	—	—	—	200	—	—	—	—
Pontosphæra Huxleyi	67,490	50,500	64,200	120,000	30,000	9,100	1,800	2,300	1,400
Syracosphæra dentata	80	100	—	600	—	—	—	—	—
Eutreptia viridis	—	—	100	6500	600	—	—	—	—

TABLE II—*Continued*

Depth in meters	1	5	10	15	25	30	40	50	60
Temperature, ° C.	15.45	15.47	15.39	13.92	8.67	8.47	8.44	8.21	8.07
Salinity, ‰	33.12	33.15	33.54	33.27	32.51	32.53	32.56	32.56	32.60
Oxygen, cc. per liter	6.18	6.44	6.30	6.58	6.40	6.38	6.24	6.23	6.33
Oxygen, % saturation	107	112	110	111	97	96	94	93	94
PO_4', mg. per cu. m.	28	31	28	49	65	68	76	72	75
NO_3', mg. per cu.m.	14	11	8	9	53	54	67	62	62
Number cc. examined	25	10	10	10	10(25)	10	10	10	10
Ceratium longipes	—	—	—	—	20	—	—	—	—
" tripos	80	—	—	—	—	—	—	—	—
Exuviælla baltica	120	300	200	—	—	—	—	—	—
Gonyaulax Tamarensis	—	100	—	—	—	—	—	—	—
Gymnodinium Lohmanni	480	300	400	200	100	—	—	—	—
" sp.	320	700	100	1500	300	—	—	—	100
Minuscula bipes	120	—	—	—	—	100	—	—	—
Oxytoxum gracile	440	200	200	800	—	—	—	—	—
Peridinium triquetrum	—	—	—	100	—	—	—	—	—
" trochoideum	—	—	100	—	—	—	—	—	—
Acanthostomella norvegica	—	—	—	100	—	—	—	—	—
Labœa conica	80	—	100	—	—	—	—	—	—
" emergens	—	—	100	100	—	100	—	—	—
" reticulata	80	200	—	—	—	—	—	—	—
" strobila	400	400	—	—	—	100	—	—	—
Lohmanniella oviformis	—	—	—	200	100	200	—	—	—
" spiralis	—	—	—	—	—	—	—	—	—
Mesodinium rubrum	360	200	100	—	—	—	—	—	—
Woodania conicoides	120	—	—	—	—	—	—	—	—
Bodo marina?	520	1800	700	1600	100	800	400	100	400
Forms not identified	640	1000	1500	2000	—	600	600	100	200

atoms and *Ceratia* were the predominant forms and *Pontosphæra* was recorded only in comparatively small numbers; while in 1933 there was a predominant *Pontosphæra* vegetation. (The temperature of the surface layers was as high in 1932 as in 1933 and the salinity even higher.)

Gran (1933) recorded a poorer vegetation in the eastern part of the Gulf than further west. Since the observations were not taken in precisely the same locality, local differences in the plankton distribution may account for a part of the differences which have been recorded in the plankton of the two years; but we should hold it likely that yearly fluctuations in the composition of the phytoplankton are still more important in producing the state of affairs which has been observed.

TABLE III

Comparison of observations at stations 1722 and 1723

Station	Depth	Temp.	Salinity	O_2	Guinardia	Detonula	Pontosphæra
	meters	° *C.*	‰	*% sat.*	*cells/l.*	*cells/l.*	*cells/l.*
1722	1	13.17	32.07	107	—	200	146,000
	6	13.04	32.07	105	—	100	105,400
	12	12.52	32.09	108	—	—	198,000
	18	11.39	32.11	109	—	200	308,000
	30	10.46	32.19	107	—	100	130,200
	42	8.50	32.42	99	—	—	13,200
	54	7.01	32.63	94	—	200	9,500
	66	6.59	32.75	92	—	60	6,460
	78	5.77	32.80	88	—	—	1,880
1723	1	15.45	33.12	107	—	400	67,400
	5	15.47	33.15	112	100	600	50,500
	10	15.39	33.54	110	—	600	64,200
	15	13.92	33.27	111	700	1300	120,000
	25	8.67	32.51	97	1800	200	30,000
	30	8.47	32.53	96	5300	—	9,100
	40	8.44	32.56	94	3700	—	1,800
	50	8.21	32.56	93	3900	100	2,300
	60	8.07	32.60	94	2200	—	1,400
	70	7.98	32.60	94	3600	—	2,800

For Georges Bank there is a similar difference in the quality of the phytoplankton. In 1932 there was observed here a rich *Rhizosolenia* vegetation with *Rh. alata* as the dominant species. This was then recorded in great numbers throughout the water column, with a maximum of 50,000 cells per liter at 25 meters. This species was also recorded in fairly large numbers from all the stations over the Bank, although it was not so abundant at the two outer stations. In 1933 at Station 1723 *Rhizosolenia alata* was recorded with a maximum number of only 300 cells per liter. One might seek the explanation for the variation in the occurrence of this species in the fact that this station was located a little further out than the stations of the previous year. However, the abundance of *Guinardia flaccida* at Station 1723 seems to be a good indication that the station was located within the Bank area. The previous year *Guinardia* was also found in the deeper samples from all the stations over the Bank, while it was not observed in the samples from the deeper part of the Gulf (Gran, 1933, p. 177).

These few observations suggest that there exist considerable fluctuations in the composition of the phytoplankton of the waters of the Gulf of Maine and adjacent areas in the summers of different years, perhaps not so much in a purely qualitative respect as in the relative abundance of the various species. The observations on net haul material have given similar indications for the larger forms (Bigelow, 1926). These variations certainly are tied up with changes in the hydrographical conditions from one year to another. For example, the occurrence of *Guinardia flaccida* solely on the Bank (and only in the deeper layers) suggests dependence upon fairly turbulent waters. The question of the relationship between the phytoplankton production and the hydrographical conditions is, however, so complex that we shall not consider it further, since the data for only two stations would not suffice as a basis for discussion. At least two years of intensive simultaneous investigation of the hydrography, the plankton, and the various environmental factors would seem necessary in a study of the influence of the water movements on the supply and conditions of growth of algæ and an explanation of yearly fluctuations. A consideration of the relationship between phytoplankton production and hydrography, especially in regard to the seasonal changes in the phytoplankton occurring in the Gulf of Maine and the Bay of Fundy will appear shortly (Gran and Braarud, 1934).

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GERM CELLS AND SEX DIFFERENTIATION IN LEBISTES RETICULATUS

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In this paper there is presented a condensed account of the results of a series of studies begun in our laboratory three years ago which deals chiefly with the history of the germ cells in *Lebistes reticulatus* and the correlation of that history with the secondary sex characters. In so far as the results closely parallel those obtained on other fish, the account will be found to be much abbreviated. A brief summary of literature dealing with *Lebistes* is also included.

Summary of Literature of Lebistes reticulatus

Lebistes reticulatus, known to tropical fish fanciers as the "guppy," is proving to be valuable material for various types of biological investigations. This species is a native of Venezuela, Trinidad, the Barbadoes, and Leeward Islands. It has been introduced into various tropical and subtropical countries as a means of mosquito control. It seems to have been first classified as *Pœcilia reticulatus* by Peters (1859), and Fillippi (1861) first included it in the genus *Lebistes.* Since then it has been assigned to various genera (*Girardinus, Acanthophacelus*) and is now known as *Lebistes reticulatus* (Regan, 1913). Regan classified it in family 294, the Pœciliidæ, and Jordan (1923) placed it in family 290, the Cyprinodontes. It seems to have made its first appearance as an aquarium pet in Germany in about 1905 (Wichand, 1906).

The brilliant color variations of the male have made it suitable for genetic investigations and the marked sex dimorphism has suggested its use for problems concerned with the determination and physiology of sex. The genetic studies were initiated by Schmidt (1919, 1920) and continued in a series of papers by Winge (1922*a*, 1922*b*, 1923, 1927, 1930, 1932) and by Blacher (1926*a*, 1926*b*, 1927, 1928) and by Eloff (1932).

These studies established the existence of a large number of genes carried by the Y chromosome, a few limited to the X chromosome, some which showed crossing over between X and Y, and one which is autosomal. A brief review of the earlier papers is given by Goodrich (1929). On account of the unusual situation of the great preponderance of sex-linked genes, Demerec (1928) suggested that the genes might be carried in a chromosome complex rather than in a single Y chromosome, but there has so far been found no cytological confirmation of this suggestion. Independent studies on the sex ratios by Huxley (1920) had led to the deduction that the female was homogametic and the male heterogametic which was, as seen above, confirmed by genetic analysis.

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[1] All work on postnatal stages and correlation with secondary sex characters has been done by C. M. Flynn.

Winge (1927, 1932) considered that his genetic results pointed to the conclusion that in *Lebistes* the male sex is determined by a single dominant gene carried by the Y chromosome and absent in the X chromosome. This theory, however, has been criticized (see Witschi, 1932, p. 206).

The cytology of *Lebistes* has been investigated by Winge (1922*a*), Vaupel (1929), and Iriki (1932), who find a haploid count of 23 chromosomes.

Breder and Coates (1932) have made an interesting investigation of the sex ratios of *Lebistes* when confined in aquaria and include precise data in regard to various details of the life cycle. Chambers and Harvey (1931) have used *Lebistes* as material for the study of ultrasonic waves.

Ginsburg (1929) gives an account of the development of the color pattern. We know of no other work on the development of *Lebistes* except the abstract of a paper read by title only (Dildine, 1933). As far as we can judge, our paper is concerned chiefly with stages earlier and later than those studied by Dildine but otherwise confirms his results.

Lebistes reticulatus is one of the easiest of tropical fishes to rear and methods of handling it are now known to all fish fanciers. Excellent accounts of methods have been given by Winge (1927) and Eloff (1932).

METHODS

Since *Lebistes* is viviparous, the embryonic stages are obtained by removal from the body of the female. Females were first selected at random from a mass culture, but in obtaining early stages it was found more practical to isolate individual fish and open each at known intervals after they had given birth to young. Because a single mating may provide sperm for four or five litters (Breder and Coates, 1932), it is not usually necessary to wait for a second pairing. Even under these conditions there is considerable variation in the age of embryos. Bouin's fluid was the principal fixative used except for very early stages when it was not practical to remove the embryo from the yolk. Under these conditions variations of Slifer's modification of Petrunkevitch's method (Slifer, 1933) were employed. Embryos were passed rapidly through the alcohols and cedar oil was used as a clearing agent. Staining was done chiefly with Heidenhain's iron hematoxylin and eosin. The very early stages were first totally stained in Delafield's hematoxylin for purposes of orientation and later destained. Altogether, sections suitable for study of about 100 eggs and embryos have been obtained. Measurements of early stages are from fixed eggs and those of later stages from living embryos. The measurements of later stages have been made from tip of head to base of caudal fin.

TABLE I

Counts of Germ Cells

Embryo	Length	Number of germ cells	Embryo	Length	Number of germ cells
	mm.			*mm.*	
1	.5	40	15	3.2	61
2	.6	52	16	3.6	192
3	.6	61	17	3.7	107
4	.7	37	18	4.2	223
5	.8	62	19	4.8	305
6	1.0	65	20	4.9	300
7	1.0	54	21	5.2	318
8	1.0	67	22 ♀	5.9	196
9	1.3	64	23 ♂	6.0	393
10	1.5	57	24 ♀	6.3	183
11	1.6	63	25 ♂	6.5	508
12	2.3	66	26 ♀	6.6	627
13	2.5	52	27 ♂	6.7	317
14	3.1	66	28 ♂	7.0	332

THE PRIMORDIAL GERM CELLS

It has been possible to identify the primordial germ cells and to follow their history from the stage of the early embryonic shield until they become finally located in the gonads. They are of large size and of oval shape, averaging about 14 μ × 10 μ, although the greatest dimension may reach 20 μ. The nucleus is large, often bilobed, with a chromatin network less dense than that found in somatic cells. The nucleolus is variable. After fixation in Bouin's fluid and Carnoy Lebrun fluid the nucleus is closely surrounded by a condensation of cytoplasm. Our observations have convinced us of the actuality of this part of the history of the germ cells. The problem of their relation to definitive germ cells is discussed below.

Counts of the number of germ cells present at various stages are shown in Table I. The difficulty of certainly identifying parts of the same cell when divided in sectioning has introduced an error into the counts, especially of the later stages. The earliest counts from the embryonic shield show about 40 germ cells, and there is very little increase until the 3.5-mm. embryo is reached, when there is a period of rapid multiplication followed by a gradual increase through later stages. Sex differentiation of the gonads is recognizable at about 5.5 mm., shortly before hatching. The sex is not, however, indicated by a relative increase in the number of germ cells of the female, as in *Platypœcilus* (Wolf, 1931), but by the increased size of the oögonia.

GENERAL FEATURES OF EMBRYONIC DEVELOPMENT

The egg of *Lebistes* averages about 1.7 mm. in diameter. It is a typical telolecithal teleost egg having meroblastic cleavage. Our sections of the cleavage stages indicate that the early blastodisc has a diameter of about .4 mm., covering about one-thirteenth of a circumference of the egg. Gastrulation involving the formation of the embryonic shield takes place when the blastoderm is about 1.3 mm. in diameter, covering one-fourth of a circumference of the egg. The medullary plate is soon laid down and an embryo of from 8 to 10 somites and of 1 mm. in length is found when the blastoderm has covered only one-half of the yolk. The circulation is established by the time the blastopore closes, when the embryo has 20 somites and is 2 mm. in length. The period of gestation is 28 days at 25° C. (Breder and Coates, 1932) and the young are born when 6.5 mm. in length and with the yolk sac already absorbed.

EXPLANATION OF PLATE I

FIG. 1. Posterior portion of a sagittal section of an early embryonic shield showing eight germ cells in the mesentoderm. 614 ×.

FIG. 2. A reconstruction of an embryonic shield showing the position of forty germ cells. 61 ×.

FIG. 3. Eight-somite stage showing the number and position of germ cells in the lateral mesoderm. 20 ×.

FIG. 4. Fourteen-somite stage showing the number and position of germ cells in the mesoderm under the somites. 27 ×.

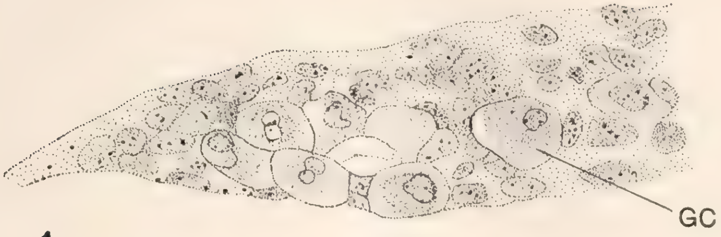
FIG. 5. From a cross-section of a 2-mm. embryo at the time of blastopore closure showing four germ cells ventral to the Wolffian ducts. 614 ×.

FIG. 6. A cross-section of a testis from a 6-mm. embryo showing spermatogonia. 614 ×.

FIG. 7. A cross-section of an ovary from a 6.4-mm. embryo showing oögonia. 614 ×.

Abbreviations: *GC*, germ cell; *Int*, intestine; *Oog*, oögonia; *spg*, spermatogonia; *St*, stroma cells; *WD*, Wolffian ducts.

PLATE I



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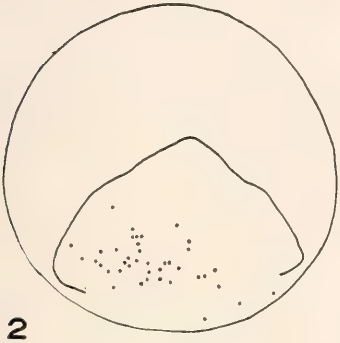

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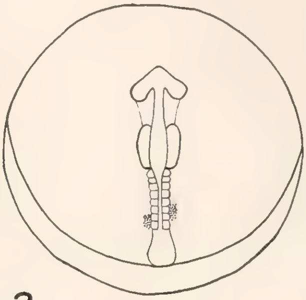

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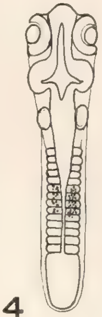

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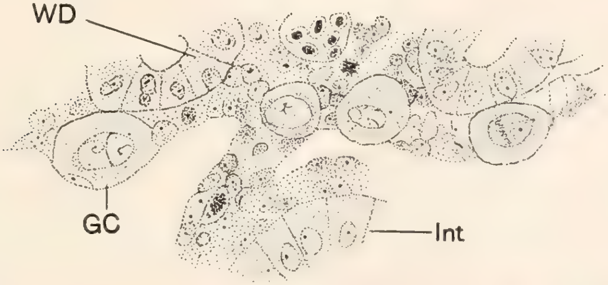

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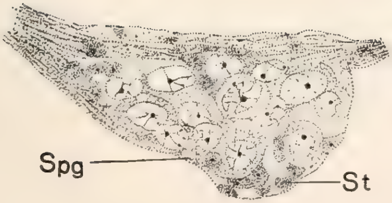

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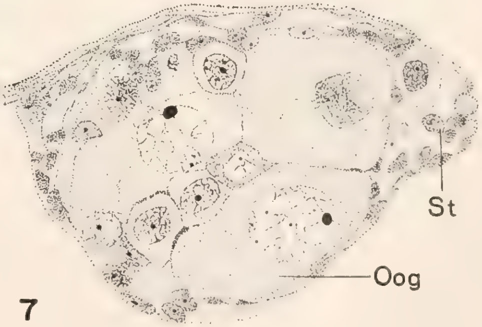

7

Description of Stages in Development

Embryonic Shield

The earliest stage in which it has been possible to distinguish germ cells is that of an early blastoderm (1.3 mm. diameter), which has an embryonic shield measuring .5 mm. along the sagittal axis and .72 mm. in greatest breadth. Before this period the identification of germ cells is uncertain as all cells are nearly of the same size. This stage is represented in Fig. 2, which is a reconstruction based on serial sections. In this are shown the approximate locations of the 40 germ cells which have, according to our criteria, been satisfactorily identified. It will be noted that the germ cells are widely and also asymmetrically distributed. These cells are in the underlying (invaginated) or mesentodermal layer (Fig. 1). Some degree of inequality of distribution of germ cells on either side of the embryonic axis has been observed up to and including the 14-somite stage, after which time they move to a median position.

In a somewhat later stage, in which the medullary plate is clearly defined, but before somite formation, the germ cells were found to be disposed in a U-shaped configuration with the base in the posterior median region and with either arm extending laterally to the medullary plate. It appears that as the medullary plate thickens and forms posteriorly it separates the cluster of germ cells into two groups.

Embryo of 1.26 mm.—Eight Somites (Fig. 3)

As the somites develop, the germ cells are found to be lying in nests in the mesoderm lateral to the somites. In the embryo figured there were about 44 germ cells which were somewhat asymmetrically disposed. Twenty-four were counted on the right side and twenty on the left side and the group on the right side extended further anteriorly than that on the left side. We have no observations which indicate that germ cells migrate through somites, as observed by Dodds (1910) on *Lophius*, but rather they seem to be pushed laterally first by the medullary plate and later by the formation of somites. The situation in this stage is closely similar to that described by Wolf (1931) for a 1.6-mm. embryo of *Platypœcilus.*

Later Stages

The later stages so closely parallel those of *Platypœcilus* that we are omitting detailed description of the individual embryos. Our observations are, however, based on the study of 16 embryos (listed in Table I) of stages from 2.5 mm. to 7 mm. in length.

During this period the germ cells first aggregate (2.7 mm.) medially ventral to the level of the Wolffian ducts and dorsal to the gut. Figure 5 shows a cross-section of an intermediate stage in the migration of germ cells (in an embryo of 2.0 mm.) and Fig. 4 an outline drawing of a 14-somite (1.5-mm.) embryo. Shortly after (3.1 mm.), the median strand of germ cells divides and they enter the newly-forming genital ridges (*cf.* Wolf, 3.5-mm. stage).

The development of the swim bladder now separates this region from that of the Wolffian ducts, which remain dorsal to the swim bladder while the genital ridges are ventral. In a 3.6-mm. embryo the genital ridge has become largely separated from the body wall and may be termed an indifferent gonad.

The period of gonadal differentiation occurs shortly before birth in embryos of about 6 mm. in length. Our few counts of germ cells (Table I) do not indicate, as in *Platypœcilus,* that at this stage there are more germ cells in the female than in the male. The chief criterion is the larger size of the germ cells in the ovary as contrasted with those in the testis. Also the nucleolus of the oögonia is usually eccentric and the chromatin threads stain more heavily than those of the spermatogonia (Figs. 6 and 7).

Female Gonad

The development continues much as described for *Platypœcilus.* The central region of the gonad becomes filled with stroma cells among which the ovarian cavity appears a few days after birth (7-day, 7-mm. fish). The two ovaries gradually approach and fuse and external indications of the double origin disappear at about 35 days after birth. In a 56-day specimen the ovarian cavity is lined with columnar epithelium among which are found oval cells with dense circular nuclei. These are similar to those which Wolf considers to be transitional stages between the epithelial cells and germ cells. There occurs no condition of complete disintegration of primordial germ cells prior to formation of the definitive germ cells such as that described by Essenberg (1923) for *Xiphophorus.*

The Mature Ovary

In the mature ovary the ovarian cavity is central, traversing its entire length, and is continued posteriorly as the oviduct opening into the urogenital sinus. The fully-developed ova extend from the surface to the ovarian cavity and are surrounded by a sheath of follicle cells.

Male Gonad

The spermatogonia do not increase in size as do the oögonia and in the nuclei the nucleolus is usually central and chromatin threads are fine and lightly staining. At first the germ cells are relatively uniformly distributed in the gonad among the stroma cells but soon (6.9 mm. at birth) the spermatogonia tend to migrate to the periphery. Later (8.2 mm., 21 days after birth) the sperm ducts appear as narrow slits surrounded by the stroma cells. The gonads are also now connected by a bridge of stroma cells. At about 36 days after hatching (9.5 mm.), the primary spermatogonia have begun to divide to form nests of cells which, when surrounded by connective tissue sheaths, form the earliest stages of the typical cysts of the mature testis. At no time have we discovered stages indicating that stroma cells or epithelial cells of the sperm ducts transform into germ cells. In this respect our findings accord with those of Wolf on *Platypœcilus* but contrast sharply with the observations of Essenberg on *Xiphophorus*.

The 48-day stage (12.8 mm.) is of especial interest because this is the period of the first appearance of external secondary sex characteristics. Sections of the testis show a marked increase in the number of cysts. Spermatogonia are in frequent division. Growth stages are abundant and a few spermatocyte divisions have been observed. No mature sperm are present. The sperm ducts are well-developed and cuboidal cells forming their walls are clearly defined. Branches of the ducts extend into the body of the testis.

In a 61-day specimen (14 mm.) spermatocytes and Sertoli cells are present but no spermatozoa have formed.

A 72-day specimen (15 mm.) shows all stages of spermatogenesis and a few of the cysts containing mature sperm have moved into the anterior sperm ducts, there becoming spermatozeugma or spermatophores.

The Mature Testis

The testis is a whitish bilobate body some 2 to 3 mm. in length, located in the dorsal posterior part of the body cavity ventral to the swim bladder and anterior to the cloaca. The two branched internal ducts unite to form a short single duct which opens into the urogenital sinus. These internal ducts are lined by a cuboidal epithelium. We find no evidence that portions of the duct separate from it to form acini as described by Essenberg (1923). Spermatophores are found at various points in the lumen of the sperm ducts.

From the above observations the progress of spermatogenesis may be summarized as follows: The primary spermatogonia at the periphery of the testis by division give rise to nests of secondary spermatogonia which become surrounded by connective tissue sheaths derived from the stroma cells. Formation of new peripheral cysts forces the earlier ones inward. During this period the various phases of spermatogenesis take place within a given cyst until it becomes filled with mature spermatozoa and the Sertoli cells are located at the periphery. The cyst is then forced into the sperm duct, becoming a spermatophore.

A special search for interstitial cells has been made at the various stages. They are, however, relatively scarce and there does not appear to be any clear correlation between the abundance of interstitial tissue and the appearance of secondary sex characters. Previous work on fish (see review by Rasmussen, 1928) shows that the interstitial tissues apparently take no such important place in the secretion of hormones as appears to be the case in mammals.

Discussion of the Germ Cell Cycle

The problem of the first differentiation of the primordial germ cells in *Lebistes* is perplexing because of the fact that when first recognizable they are widely scattered in the mesentoderm. This distributed condition does not readily fit into theories of embryonic segregation or induction. It may be supposed that there exists an embryonic segregate prior to gastrulation and that the cells then determined have become widely scattered in the spreading of the blastoderm. Even so, however, the determination is most likely to be of the labile or reversible type because, if we accept the results on induction of twins in other fish (*cf.* Stockard, 1921), it appears that any part of the germ ring can give rise to a complete embryo. Under these conditions only those of the potential germ cells which happen to enter the embryonic shield ultimately become the irreversibly determined primordial germ cells.

The later history of the germ cells until the period of formation of the " definitive " germ cells is quite in accord with accounts of the conditions in other fish. In regard to the moot question of the relation of the primordial germ cells to the definitive germ cells, we can only say that our observational evidence indicates clearly that primordial germ cells do give rise to definitive germ cells. The evidence of transformation of other cells (stroma cells) into definitive germ cells is inconclusive. Cells which might be considered transitional have been discovered in the ovary but not in the testis. We realize that this question can only be solved by experimental methods such as those utilized by Heys (1931) and Butcher (1932) which are not readily applicable to our material.

The Secondary Sex Characters

In *Lebistes* the two sexes are externally indistinguishable at birth. At maturity the female is distinctly larger than the male, averaging about

30 mm. in length from tip of snout to the base of the caudal fin, while the male averages 18 mm. in length. The female is of an olive brown color with the edges of the scales somewhat darker, producing a net-like effect—hence the specific name *reticulatus*. Refraction of light from the side of the body may give violet and silvery effects. Fins are colorless except the caudal, which may be yellowish or light green at the basal part.

The male is brilliantly colored, varying according to the genetic constitution (see colored plates, Winge, 1927). In addition to the brilliant spots or areas of red, blue, green, etc., there are a number of paired black body spots in phenotypes of our stock (*cf.* Winge, 1927). These latter are the markings of the male which appear first in development preceding any other recognizable sex character. In the male the anal fin is developed to form an intromittent organ or gonopod.

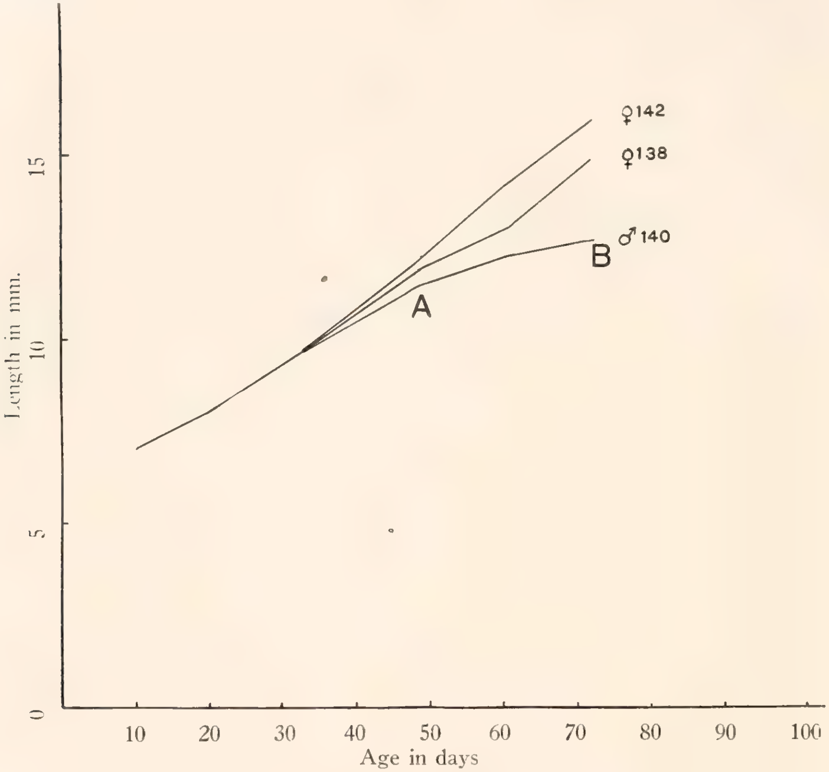

FIG. 1. *A*. Time at which black spots appeared and gonopod became elongated in ♂ 140. *B*. Time at which colors appeared and gonopod was well developed in ♂ 140.

The size relations and development of black spots were especially followed in the attempt to establish a correlation between the development of the gonads and the secondary sex characteristics.

Fish were anesthetized, examined, and measured at 10-day intervals and from these lots individuals were taken for study of the gonads. All were kept in similar aquaria and under identical temperature conditions. It was usually necessary to keep fish isolated for identification. Isolation, however, appears to retard the growth rate. They thrive better when a number are reared together. If food is discovered by one in a group, the active motions bring other fish to the source of supply; otherwise it often remains undiscovered. Other factors may be involved (*cf.* Allee, 1931). On this account our records may deal with a somewhat retarded development.

A graph (text fig. 1) shows a typical record of three fish. It will be noted that the divergence in size occurred in this case at about 35 days after birth and the black spots on the male and beginning of elongation of the gonopod at 48 days. Sometimes there are size differences at birth, the female being slightly longer, but the growth of all is at nearly the same absolute rate until a point of divergence begins between 30 and 53 days of age. The black spots were first detected at 48 days but sometimes not until the sixtieth day. The elongation of the gonopod was also first noticed at 48 days but does not become marked until about 60 days. Other colors develop more slowly, first appearing at from 60 to 70 days. Full functional maturity of males was attained at from 80 to 100 days, while females bear their first litter between 90 and 120 days. If 28 days is allowed for gestation, this points to a similar or slightly earlier period of maturity than in the male. Inadequate feeding, as observed in the care of some thirty individuals, will markedly retard both the growth and the appearance of the black spots in the male.

If the data in regard to size and other secondary sex characters are compared with that in regard to the development of the gonads, it will be found that in the male there occurs a period of rapid spermatogonial multiplication beginning at about 36 days and continuing until after the 48-day period, when secondary sex characters appear.

At the corresponding period in the female there is no rapid multiplication of oögonia. They are chiefly increasing in size. In neither case is there any marked development of interstitial cells.

These results in regard to the relation between the testis and secondary sex characters are in accord with the general results on fish. Rasmussen (1928) and Moore (1931), who have summarized the evidence, point out that the conditions in fish have not been found uniform. In some fish (Courrier, 1921, 1922) there appears to be an increase of interstitial cells at the breeding season when nuptial colors appear, but in other cases no such correlation has been found. There is some evidence, however, that the presence of the testis as a whole does affect a sex coloration. Kopec (1927) has concluded from his castration experiments that the presence of the testis was necessary for the development of nuptial colors, and Blacher (1926*b*) points out that in cases of sex reversal from male to female in *Lebistes* there is testicular atrophy.

SUMMARY

1. The history of primordial germ cells in *Lebistes reticulatus* has been traced from the early gastrula (embryonic shield) until they become definitive germ cells in the ovary and testis.

2. When first observed, the primordial germ cells are scattered irregularly in the mesentoderm.

3. The formation of the medullary plate separates the germ cells into two lateral groups which become again more widely separated by the formation of somites and lie in the lateral mesoderm.

4. By the 2.7-mm. stage the germ cells are located in a median strand dorsal to the gut. Slightly later (3.1 mm.) they move laterally to the genital ridges.

5. Visible sex differentiation of gonads occurs shortly before birth (6 mm.).

6. The evidence indicates that primordial germ cells give rise to definitive germ cells. There is slight evidence that stroma cells may also give rise in the female to definitive germ cells. No such evidence has been found in the male.

7. Divergence in size between the sexes occurs about 35 days after birth and the first appearance of markings on the male at 48 days. This is coincident with a period of rapid multiplication of spermatogonia and progress of growth stages of spermatogenesis. No correlation with multiplication of interstitial cells has been observed.

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OBSERVATIONS ON REPRODUCTION, PREMATURATION, AND FERTILIZATION IN SABELLARIA VULGARIS

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INTRODUCTION

Sabellaria vulgaris (*cf.* Verrill and Smith, 1874) is a polychæte annelid living in sand tubes on dead *Echinarachnius* and other shells, in Bryozoa nodules, and occasionally on stones. The animals occur apparently in considerable numbers in the vicinity of Woods Hole, Mass., and eggs and sperm may be readily obtained throughout the summer months. Shedding takes place immediately following the removal of the animals from their tubes.

Two European species of *Sabellaria* (*alveolata* and *spinulosa*) have been studied by Fauré-Fremiet (1924), Hatt (1931), and Dehorne (1910*a, b,* 1911, and 1913). In addition to describing the origin, mechanics of shedding, prematuration, and fertilization of the gametes of *S. alveolata,* Fauré-Fremiet has analyzed the egg chemically and has provoked artificial parthenogenesis by increasing the alkalinity and elevating the temperature of the sea water. Hatt has studied the organization of the egg of the same species by isolation of blastomeres and cutting experiments, while Dehorne has been especially interested in the behavior of the chromosomes during maturation in the egg of *S. spinulosa.*

No reference has been found in the available literature to any study of the American species, *Sabellaria vulgaris.* For this reason it has seemed of interest to follow the early development of this species, from the time of the separation of the ova from the ovaries to the early cleavage stages, with special reference to longevity of the gametes, fertilization reaction, and the relation of the first cleavage plane to the sperm entrance point and to the position of the polar bodies. In many respects the early development of *S. vulgaris* is strikingly similar to that of *S. alveolata* as described by Fauré-Fremiet. This animal was called to my attention by Professor Earl A. Martin.

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[1] Contribution No. 9.

SHEDDING OF THE GAMETES

Figures 1 and 2, Plate I, are photomicrographs showing the position of the gonads and the presence of gametes in the cœlomic cavity. In Fig. 2 the ovary may be seen as a dark cellular mass to which young ova of various stages of development are attached by narrow stalks, and the cœlomic cavity contains a few irregularly shaped detached ova with large germinal vesicles. As the young ova develop they become separated from the ovary and are stored in the cœlomic cavity which they greatly distend. Extrusion occurs through minute pores (one of which is present in the photograph) in the lower lateral wall of the segment (compare *alveolata*).

The irregular shape of the ova (Fig. 4, Plate I) is said to be caused by crowding in the cœlomic cavity, and, with release of pressure following shedding, the spherical form is attained. In addition to this change of shape, the difference in pH of the sea water from that of the cœlomic cavity results in the elevation of the vitelline membrane and the initiation of the prematuration changes. During this sequence of changes the original point of attachment of the ovum to the ovary disappears.

The normal shedding of *Sabellaria vulgaris* is said by Verrill and Smith (1874) to occur during May and June, but this has not as yet been verified. At other times the release of the worm from its tube is the stimulus for shedding to begin and this continues until most of the eggs have been extruded. A quiescent animal which has ceased shedding may be stimulated to renewed activity by a weak chlorotone solution, indicating that muscular contraction plays a part in the extrusion of the gametes. Eggs and sperm were secured up to the latter part of August, at which time the experiments had to be discontinued. Undoubtedly they could have been obtained later. Both eggs and sperm are shed in considerable quantities depending upon age and size of the animals and the number present in the cœlomic cavities. The shedding time varies between five and ten minutes and may last as long as twenty minutes.

The normal shedding time of May and June is followed by a second but shorter period extending from around August first to fifteenth. The number of fertilized eggs secured during the first part of July steadily decreases, until about the second week very few cases of development occur even though the animals shed together. A high percentage of fertilization can be secured, however, by changing the normal pH of the sea water (at Woods Hole around 8.3) to 8.6 according to the method of Lillie (1923). Around the first of August evidences of normal fertilization begin to increase and this reaches a high percentage (90 per cent or more) during the second and third week, after which the fertilizability of the ova in normal sea water steadily decreases. The change in alkalinity does not appear to affect the fertilized ova adversely provided they are removed to fresh sea water.

In contrast to the above, the natural shedding time of *Sabellaria alveolata* is August 20, after which new oöcytes may be produced to give another shedding time around September 15. Sexual maturity may occur as early as June and eggs may be secured by placing females in sea water at 10° C. All shed at the same time and the ova mature at the same rate. The same holds true for *S. vulgaris* and in this way a considerable number of eggs may be obtained at one time. Labbé (*cf.* Fauré-Fremiet, 1924) has found females of *S. alveolata* full of eggs in April which were shed freely but could not be fertilized in the ordinary way, while LoBianco describes the finding of females filled with eggs in October and states that these, on shedding, could be fertilized.

The Ovum Previous to Fertilization

Immediately after shedding, the ova are of odd, irregular shapes due to pressure in the cœlomic cavity of the female (compare Just, 1912, on *Nereis*), and a conspicuous vitelline membrane is closely pressed to the surface of the egg (compare *S. alveolata*). No micropyle is visible nor any evidence of the point of original attachment of the growing gamete. The germinal vesicle is a large, vesicular, whitish area more or less eccentrically located. As seen under the microscope, the egg is colored a light green due to the presence of a large amount of deutoplasmic substance distributed throughout the cytoplasm. There is no conspicuous colored granulation and the egg is too opaque for observation of internal processes except in the behavior of the germinal vesicle.

Visible polarity appears at once in a series of prematuration changes involving (1) the breakdown of the germinal vesicle and the flowing of its contents towards one side of the egg to form a clear hyaline cap-like area at the future animal pole, (2) a change in shape of the egg so that it takes on a spherical form, and (3) the elevation of the vitelline membrane (compare *Cerebratulus*). The vitelline membrane rises from the egg surface during and subsequent to the assumption of the spherical form. While no accurate measurements have been made, it appears that the accumulated fluid between membrane and egg is not derived from the egg cortex (*Nereis*), as no conspicuous decrease in diameter of the egg or cortical change is noted, but is the result of the change in the permeability of the membrane itself. The height of elevation amounts to about one-fourth or even one-third the diameter of the egg. As it rises the membrane is variously folded and these folds may never disappear even in very old eggs or in those that have been fertilized. The membrane may be removed by the intestinal fluids of the blue crab or with strong NaCl solution (*cf.* Hatt, 1932). Prematuration stops at the metaphase stage of the first polar spindle (*cf.* Lillie, 1906).

During its elevation and thereafter fine protoplasmic processes extend from the inner edge of the membrane to the egg surface. These taper towards the membrane and are thicker at the base. Their origin possibly lies in the localized attachment of the membrane to the egg surface and during elevation the cortical hyaline protoplasm is thus drawn out into fine streamers. These strands persist for a long time in unfertilized eggs but shorten and disappear after the spermatozoön has entered.

The elevation of the vitelline membrane, together with the formation of these fine protoplasmic processes previous to fertilization, constitute two phenomena which are not common in other ova. Chambers (1933) describes fine processes, extending between egg cortex and fertilization membrane of the egg of *Echinarachnius* and *Asterias,* which quickly disappear. If the egg of *Cerebratulus* enters the sea water, a vitelline membrane elevates to a considerable distance above the egg cortex and a micropyle (?) is present in the vicinity of the former stalk of attachment. Penetration may occur anywhere and the sperm encounter no difficulty in penetrating the membrane. The changes occurring in the egg of *Nereis* are too well known to require reviewing here. It is sufficient to note that in this case the protoplasmic strands are remnants of cortical material remaining after the extrusion of the jelly and they disappear after fertilization.

If unfertilized eggs are allowed to stand for several hours there frequently occurs a stratification of cytoplasmic components to form three zones, (1) a large dark zone, staining deeply with Nile blue sulphate, which includes oil droplets and yolk material; (2) a smaller granular zone from which the yolk material is absent, and which stains a pale blue; and (3) a clear hyaline zone. These three zones bear no fixed relationship to each other nor to the polar cap of the animal pole, but the hyaline cap formed at the breakdown of the germinal vesicle may lie at one side or form a fourth layer. The cortical clear cytoplasm is more distinct in these eggs. Another result is a lobular fragmentation of egg material that closely resembles cleavage in the earlier stages but soon becomes irregular. This is clearly a degenerative effect and will be described below in the section dealing with the effects of the change in pH. This fragmentation, occasionally preceded by the appearance of one or two polar bodies, would almost give the impression of artificial parthenogenetic development except that subsequent fragmentation becomes irregular and no swimming embryos develop.

Fertilization

No membrane elevation occurs at fertilization and the only evidence that a spermatozoön has entered, previous to the formation of the polar bodies, is the slow withdrawal and final disappearance of the protoplasmic strands. They persist for hours in unfertilized eggs. As to their function, it is conceivable that they serve to transmit the stimulus of sperm contact to the egg substance across the wide fluid-filled subvitelline space and the one attached to the spermatozoön may draw it into the egg unless it is previously engulfed by the fertilization cone. Their presence may also account for the large size and marked irregularity of the fertilization cone, since cone formation progresses along pathways previously indicated by these strands. During penetration the strands pull away from the inner edge of the vitelline membrane, shorten and thicken and frequently curl or form minute knobs at the free ends. They disappear completely by the time the first polar body forms (Figs. 5 and 6, Plate I).

Penetration may take place at any point around the periphery of the egg but this occurs more frequently in the animal hemisphere. Not every spermatozoön which touches the membrane enters the egg. They may adhere quietly to the membrane for a few seconds and then slip away to become attached to a neighboring egg, while others will adhere quietly for long intervals even after fertilization has occurred. The tails may remain motionless or very slowly whip back and forth causing the egg to vibrate slightly. Approximately thirty minutes after being introduced into the vicinity of the ova, all activity of the spermatozoa ceases and they either are attached to the egg membranes or lie motionless on the surface of the glass slide. Newly-shed eggs fail to produce any renewed activity.

The response of the egg to the entering spermatozoön is the abrupt formation of one or several hyaline exudations which fuse to form a large protoplasmic cone. Several of these cones may be initiated in a single egg but the one which reaches the membrane first is the successful one. This contact overcomes the tendency for accessory cone formation and the others are withdrawn. At first the fertilization cone contains only hyaline-cortical material, but as it enlarges its center becomes filled with endoplasmic granules. The formation of the cone thus appears as a rupturing effect caused by a lowering of surface viscosity comparable in a sense to the formation of pseudopods. The flowing of the granules into the cone may continue until the cone reaches the membrane. As the cone is withdrawn the granules slowly disappear into the egg, leaving eventually only hyaline material (text fig. 1).

During its existence the huge irregularly-shaped cone is constantly changing in shape, and during withdrawal and after the disappearance of the granules may break up into several bodies one of which may remain in contact with the sperm head. Temporary exudations from the cone itself form, become globular, and may be withdrawn as the cone grows towards the membrane. Actual penetration often involves a considerable interval, several minutes elapsing between the time the cone reaches the membrane and its final disappearance. As the cone is retracted the region of the membrane to which the spermatozoön is attached is pulled inward to form a narrow cavity containing it. Subsequently the sperm head is pulled through the membrane and in this process the head is markedly elongated. When the spermatozoön is about halfway through the membrane, the latter flips away and outward, with the tail or at least a part of it still attached. The membrane was thus under considerable tension. This shows that there had occurred a definite adhesion between membrane and cone which was sufficiently strong to enable the cone to draw the membrane inward against pressure.

In very few cases has the cone been observed actually to engulf the spermatozoön (text fig. 1). In general the latter is attached to the surface of the cone and is thus pulled into the egg (text fig. 2). Fre-

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Explanation of Plate I

1. Cross-section of a male, showing a large mass of spermatozoa in the cœlomic cavity. At the right is a small portion of the digestive tube. × 120.

2. Cross-section of a female. In the cœlomic cavity may be seen the darker ovarian mass together with detached ova. To the left is a portion of a nephridial tube containing a compact mass of ova. The animal was fixed immediately after shedding had ceased. × 120.

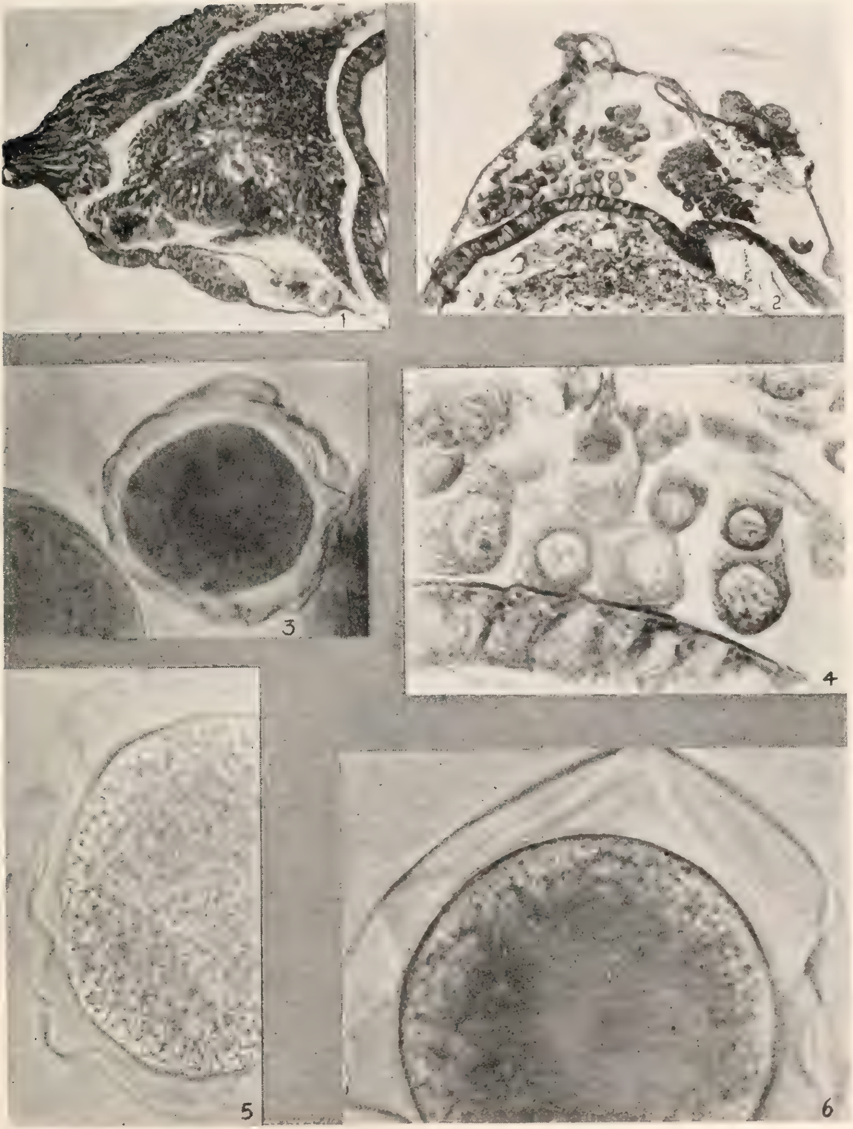
3. A living egg in sea water. The egg has become approximately spherical and the vitelline membrane elevated. × 900.

4. A portion of Fig. 2 under higher magnification to show the irregular shape and large germinal vesicle of the ovum previous to shedding. The vitelline membrane is closely pressed to the egg surface. × 550.

5 and 6. Enlarged photomicrographs of living ova taken before and after the entrance of the spermatozoön. In Fig. 6 the delicate protoplasmic strands, extending between egg cortex and the elevated vitelline membrane, are visible. In Fig. 5 the strands have disappeared, indicating that a spermatozoön has entered.

PLATE I

(All figures are photomicrographs)



quently just before its disappearance it may be engulfed or the cone may disappear entirely, leaving a thickened thread between membrane and egg surface, which draws the spermatozoön into the egg (text fig. 3). This reaction is comparable to that described by Chambers for the eggs of *Asterias* and *Nereis*. After the penetration of the spermatozoön into the egg the larger area of clear hyaline protoplasm in this area disappears and nothing remains to show where it has entered except external markers such as accessory spermatozoa or folds in the membrane.

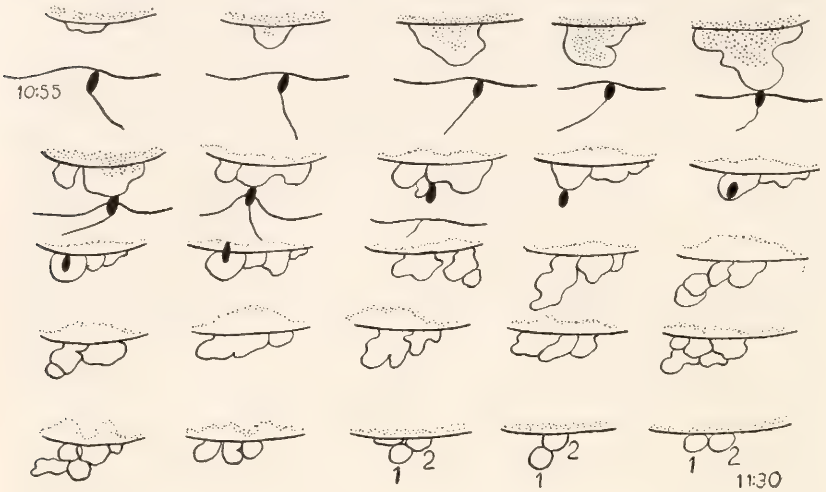


FIG. 1. A series of diagrammatic drawings illustrating the entrance of a spermatozoön into an egg of *Sabellaria vulgaris*. The time interval is indicated. After the large fertilization cone, containing endoplasmic granules, had come in contact with the vitelline membrane at the point of sperm entrance and had pulled the spermatozoön through the membrane, it broke up into several exudations, one of which engulfed the spermatozoön. The flipping out of the membrane following passage of the spermatozoön through it is shown. Following the disappearance of the spermatozoön into the egg, several temporary exudations formed in this region and by 11 : 30 two polar bodies had appeared.

In the case illustrated the thickened thread was the remnant of the fertilization cone from which the more fluid material had been withdrawn. As such it consisted of cortical material only and its withdrawal was but a final step in cone retraction. This phenomenon was seen so many times as to appear significant as a possible explanation of the origin of the fertilization thread described so frequently in the literature.

There are at least three times in the early development as far as the two-cell stage, in which a conspicuous alteration in the state of the cortical layer of the egg takes place, *i.e.*, at fertilization (cone formation and withdrawal of the protoplasmic strands) and at first and second polar body formation. According to Whitaker (1931), there occurs an alteration of the physical state of the cortical layer at the time of cone formation and its disappearance is marked by an increased viscosity of this layer. A similar change must occcur at the time of polar body formation, in addition to the division phenomenon, since not only is the surface of the egg slightly indented after the polar body is pinched off but hyaline exudations accompany this process. The reaction is not confined locally but extends unevenly outward through the egg surface to a diminishing degree. The more peripheral exudations are generally smaller than those nearer the fertilization cone or polar bodies; they seldom form around the entire egg surface; and moreover they are scattered. The cortical change is not uniform.

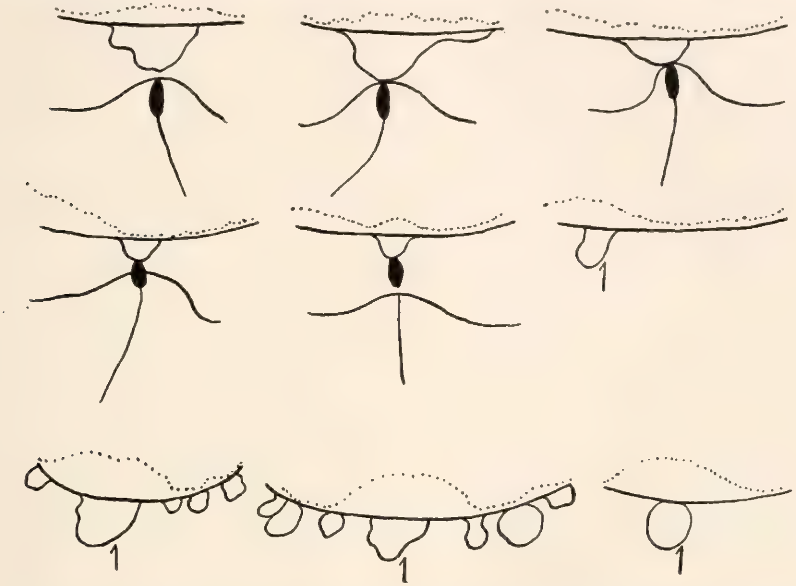


FIG. 2. Another case of fertilization in which the spermatozoön was not engulfed by the fertilization cone which was small and non-granular. The former was drawn into contact with the egg surface by the retraction of the cone. During the formation of the first polar body many hyaline exudations formed but were only temporary.

In one case the spermatozoön was seen to enter the egg at one side of the polar hyaline area in the vicinity of the maturation spindle.

Considerable activity occurred in this region as evidenced by a bubbling of the cortical area without entire disappearance of the parts of the fertilization cone. All but one of the exudations soon disappeared and this one turned out to be the first polar body (text fig. 1).

The time involved in the early developmental stages has been carefully followed in several cases. Eggs shed at 11:49 were fertilized at 11:49 by sperm shed at 9:00. The fertilization cone reached its maximum size by 11:52, after which it was retracted, pulling membrane and attached spermatozoön with it. By 11:56 it had disappeared, leaving a fine filament extending from egg surface to membrane at the point of sperm attachment. At 11:59 the other protoplasmic processes had almost disappeared. The filament continued to shorten, pulling membrane and sperm nearer to egg surface, and at 12:05 a second exudation which later turned out to be a polar body appeared at one side of the filament (text fig. 3). The spermatozoön began to penetrate the vitelline membrane at 12:04 and was through at 12:06, the membrane flipping outward as soon as the spermatozoön was through. At 12:06 it was attached to the egg surface in direct contact with the polar body. One minute later it had disappeared into the underlying

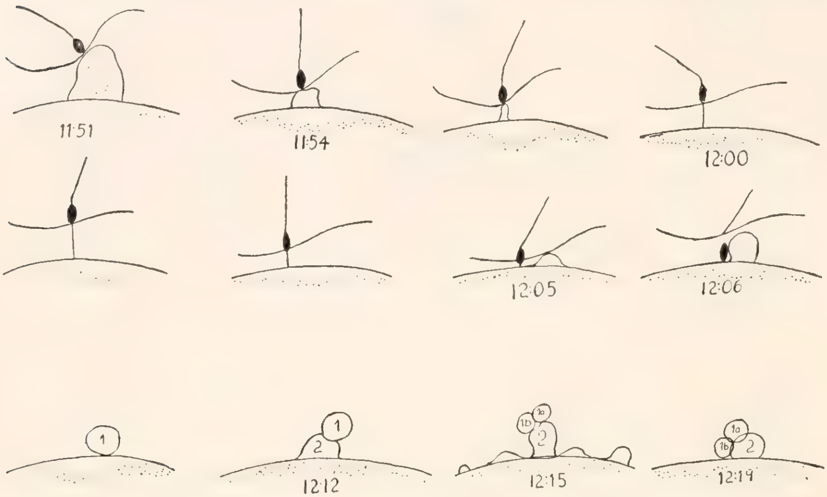


FIG. 3. In this case, the large fertilization cone disappeared, leaving a thickened thread connecting spermatozoön and egg cortex. The spermatozoön was drawn into the egg by the contraction of this thread, but before its disappearance an exudation appeared at its side. This was the first polar body which later divided into two parts. At the time of appearance of the second polar body, several hyaline exudations temporarily appeared in the vicinity.

protoplasmic cap. The second polar body appeared at 12: 12 and by 12: 15 the first polar body had divided. The polar lobe appeared at 12: 30 and at 1 o'clock 2–4 cell stages were found. This case illustrates the relative instability of the pre-maturation figure since the first polar body appeared before the spermatozoön had actually entered. Several cases of this were observed in which sperm entrance occurred in the region of the clear protoplasmic cap of pre-maturation. During the extrusion of the second polar body several exudations formed in the vicinity but the egg had resumed its spherical shape by 12: 19.

In another case eggs shed at 9: 00 were fertilized at 9: 14 with fresh dry sperm suspension. In all the studies the cover-slip was supported by pieces of No. 1 cover-slip to prevent pressure on the eggs. In this example the sperm entered before pre-maturation was completed. By 9: 25 all the protoplasmic processes had disappeared. The point of sperm entrance was marked by the presence of accessory sperm and a fold in the vitelline membrane. Meanwhile the contents of the germinal vesicle were seen to be moving towards the egg cortex along a path at right angles to the plane of sperm entrance. By 9: 37 several exudations, which lasted for one minute, appeared in the region of the clear polar cap. One remained as the first polar body. For a short interval after the polar bodies were fully formed the egg cortex was slightly depressed or indented just beneath the polar body. The second polar body formed just beneath the first and was complete by 9: 48. Exudations from the adjacent cortex again appeared at this time. At 10: 00 the polar lobe made its initial appearance opposite the region of polar body formation. Polar lobe formation is preceded by a distinct flattening of this region. At 10: 05 the polar lobe had reached its maximum proportion and the first cleavage furrow was indicated by a constriction to one side of the polar bodies. In this example the sperm entrance point bore no relation to the plane of first cleavage but there was slightly less than a 45° deviation. The first cleavage was completed by 10: 13.

The Relation of the First Cleavage Plane to Point of Sperm Entry and Position of Polar Bodies

In the above case the cleavage plane cut the egg in such a fashion that the polar bodies lay on the smaller blastomere and the larger blastomere contained the contents of the polar lobe. Two other developing eggs were in the field at the same time and in both cases a similar relationship was observed. In each case the first polar body divided to form two smaller ones. Later, however, at the two-cell stage, one or more of the polar bodies may come to lie on the large blastomere. Subsequent cleavage is more or less regular but it is not uncommon to find three-cell stages. This particular culture gave 97 per cent swimming embryos. The orientation of the first cleavage plane with reference to the positions of the polar bodies has been observed many times.

The entrance point of the spermatozoön appears to bear no fixed relation to the first cleavage plane (text fig. 4). Out of twenty cases only six showed an exact coincidence. In one case in which a spermatozoön entered the polar cap to one side of the median point, the polar bodies were pinched off beside the entrance point and the first cleavage plane passed through this point. While these observations are perhaps indicative, the number of cases followed are as yet too few for any definite conclusion. Morgan and Tyler (1930) found that in *Cumingia* the coincidence occurred in 77 cases while in 21 cases there were less than 90° deviation. In *Chætopterus* 48 cases of coincidence were observed; 35 cases deviated less than 45° and 33 cases more than 45° but less than 90°. In *Nereis* 51 per cent agreement was seen. Wilson and

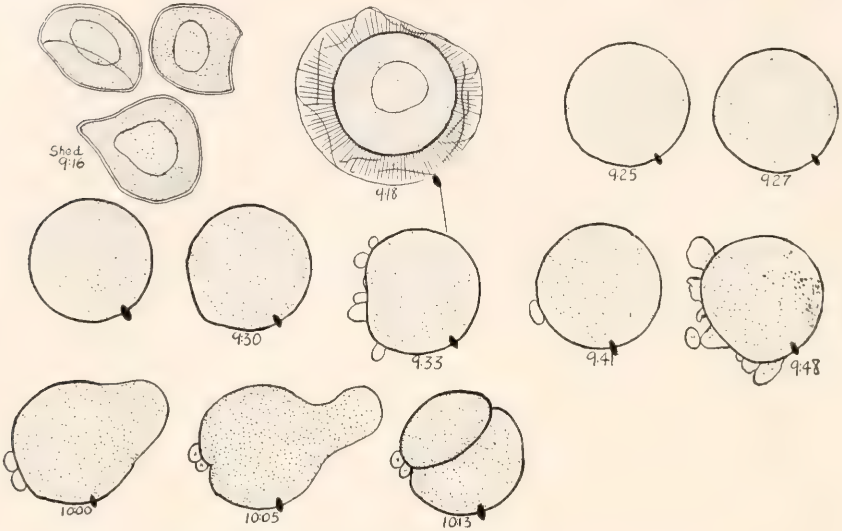


FIG. 4. A series of diagrammatic drawings made from a living ovum to illustrate the early development as far as the two-cell stage. When the eggs are shed they are irregular in shape and have large germinal vesicles. Subsequently they become spherical, and the vitelline membrane elevates. Fine protoplasmic strands which extend across the sub-vitelline space disappear after fertilization. The contents of the germinal vesicle form a clear cap at the animal pole. The plane of first cleavage bears no fixed relationship to the entrance point of the spermatozoön (black body) and passes to one side of the pole as indicated by the position of the polar bodies.

Matthews observed in *Toxopneustes* that the great majority of first cleavages passed at least approximately through the entrance point. Just (1912) found an even higher percentage of coincidence in *Nereis*. He states that the sperm entrance point determines the first cleavage plane which passes through this point. The first cleavage plane in *Sabellaria* does not pass through the pole as determined by the position of the attached polar bodies, but slightly to one side.

Length of Fertilizing Power of Eggs and Sperm

The majority of the observations made upon the process of fertilization in *S. vulgaris* involved the use of slightly alkaline sea water (8.6), as the normal breeding season, which is said by Verrill and Smith to occur during May and June, had passed and a good percentage could not otherwise be secured. What the condition is after the second but shorter shedding time (early August) is not known, as observations had to be discontinued. Alkalinity was produced by using n/4,000 NaOH in sea water. Eggs shed in chlorotone during anesthesia of the female and fertilized in alkaline sea water gave as high as 92 per cent cleavage.

The most numerous cases of successful fertilization are secured when the sperm and eggs are shed simultaneously in the same medium. This applies either to the use of a more alkaline medium or to fresh sea water. According to Fauré-Fremiet, fertilization of eggs that have stood for more than an hour is uncertain. In the present study the interval appears somewhat longer but the length of egg life is not long. In one case eggs shed at 9:00 A.M. were fertilized at 4:45 P.M. with fresh sperm. The next day no embryos had developed, 10 per cent showed lobular fragmentation, some of the remainder showed stratification, and a few were markedly swollen.

The activity of sperm does not last very long. In one case eggs shed in normal sea water at 12:00 noon (August 17) were fertilized immediately by a heavy dry sperm suspension shed at 11:00 A.M. Twenty-five minutes later no moving sperm were present in suspension but either adhered to the vitelline membranes or were non-motile on the surface of the slide. This culture gave almost 100 per cent swimming embryos. In subsequent cultures the activity of the sperm ceased 20–30 minutes after addition to the egg suspension. Freshly shed eggs added to the cultures caused no reactivity of these sperm. Eggs and sperm shed together at 9:40 gave the same high percentage of swimming embryos.

In another case (August 17) eggs shed at 8:50 A.M. were fertilized by sperm shed at 4:55 P.M. (fresh sea water). Polar bodies were slower in forming, but twenty-four hours later the culture showed approximately 98 per cent swimming embryos. During the interval previous to fertilization no cytolysis, stratification, or fragmentation occurred and protoplasmic strands were still present.

A comparison between the length of fertilizing power of the gametes in alkaline sea water and normal sea water shows some variation. Moreover, in the former case fertilization is occurring between gametes shed during a period when fertilization is not normal or occurs in a low percentage of the cases which represent animals coming late to sexual maturity. Furthermore, the results are not at all certain when alkalinity of the medium is increased slightly.

This question must be studied further, but the data available at the present time seem to show that in an alkaline medium the highest percentage of fertilization occurs when males and females shed together. As high as 98 per cent of swimming embryos have been secured in this way. Dry sperm shed an hour before addition to egg suspension fail entirely to penetrate the eggs. Between these two extremes the percentage of successful penetration decreases. Eggs one to three hours old will give around 96 per cent swimming embryos when fertilized with fresh sperm.

In normal sea water, as observed during the second breeding interval (August 15–22), the length of fertilizing capacity of the gametes is markedly increased. It has been found in some cases that eggs eight hours old gave 98 per cent swimming embryos when fertilized with fresh sperm. Fresh eggs plus sperm one hour old gave only 45 per cent success. The percentage markedly decreased with the age of eggs and sperm. Successful insemination is seldom secured to any extent with sperm more than one hour old. In general it may be said that the highest percentage of fertilization may be expected if the two sexes shed together, whether in sea water or slightly alkaline sea water—a conclusion which agrees with that of Fauré-Fremiet.

Effect of Change in pH on Egg

According to Fauré-Fremiet, the pre-maturation phenomena of the sabellarian egg is initiated by the change in alkalinity of the sea water as contrasted with that of the cœlomic cavity, and it is possible to secure artificial parthenogenesis by raising the pH to 12 or by elevating the temperature to 28° C. In the present observations it has been found that a slight elevation of pH from 8.3 of normal sea water to 8.6 will enable fertilization to occur where normally it does not to any extent;

i.e., from early in July to around the first of August. This increase in alkalinity will not alone induce development nor does it appear to harm the eggs provided they are removed to fresh sea water after the spermatozoa have entered. In some instances, when the fertilized eggs remained in the solution, the polar body formation and the first few cleavages occurred in a typical fashion. Subsequently, however, the cleavage became irregular, some eggs which had formed polar bodies failed to develop further, and no embryos formed. However, other experiments gave a high percentage of swimming embryos.

A few experiments were made involving the effect upon the unfertilized egg of changes in pH. Eggs of the same female were used throughout the series. In some instances the female was allowed to shed for a certain time in one solution and then removed, quickly dried with blotting paper, and transferred to the next solution; this continuing until all the solutions contained eggs. The pH of the various solutions was tested by B. D. H. Universal Indicators and as such are only approximate.

Series A. After one-half hour at a pH of 11, some eggs showed shortened processes or total absence of these photoplasmic strands. Pre-maturation and membrane elevation were typical. After one and one-half hours the eggs were transferred to normal sea water. One hour later out of 55 eggs, 14 showed polar body formation and 2 were at the two-cell stage. Four and one-half hours later 96 per cent showed abnormal cleavage or balls of cells. Twenty-four hours later no embryos had formed and disintegration was far advanced. At a pH of 10.5 fewer showed polar body formation and abnormal cleavage. At a pH of 10 much the same result was seen. With pH of 9.5, 9, 8.6, 8.3 fewer showed polar body formation and abnormal cleavage in the given time. But twenty-four hours later all had fragmented. No embryos developed in any case and disintegrative effects were conspicuous. In the control culture 97 per cent cleaved normally and formed embryos. The latter were eggs which had been fertilized in slightly alkaline sea water (8.6) and then transferred immediately to normal sea water.

This experiment was repeated several times with variations in the length of exposure. In every case the final result was the same (*i.e.,* abnormal cleavage followed by death), although in the solutions with the greater alkalinity the number of eggs showing preliminary polar body formation was slightly higher. Frequently the polar bodies were of varying sizes and occasionally fragmented.

As these preliminary results indicate, no cases of true activation were secured which formed embryos. The initial stages such as retraction and disappearance of the protoplasmic strands and the formation of the polar bodies compared closely with similar phenomena in fertilized eggs although somewhat delayed. It would appear from this that a change of pH is capable of initiating profound change in the egg immediately after shedding but that at the same time the exposure leads to death.

The instability of the pre-maturation spindle is further shown by the effect of vigorous shaking in a test tube. Unfertilized eggs shaken from one to five minutes behaved in a way comparable to the above; *i.e.,* one or two polar bodies formed, the first few cleavages were frequently more or less regular, but no swimming embryos developed. The earlier cleavages soon became irregular, giving an effect of degenerative fragmentation, since on sectioning many of the cytoplasmic fragments were without nuclear material. Unfertilized eggs from the same original batch left in sea water underwent fragmentation and in many cases the egg contents were stratified into three zones.

Eighty per cent hypotonic sea water failed to cause polar body formation. Eggs were put into this solution forty-five minutes after shedding and were removed to fresh sea water at one-minute intervals thereafter up to fifteen minutes. Other dilutions of sea water gave a similar result.

The observations reported above are of a preliminary type intended primarily to determine the availability of the egg for experimental work. As such they would seem to show that the egg of *Sabellaria vulgaris* offers possibilities for work upon a species that has not as yet been studied to the extent of others common to Woods Hole.

Summary

1. *Sabellaria vulgaris,* a polychæte annelid inhabiting sand tubes on dead *Echinarachnius* and other shells in Bryozoa nodules, and on stones, occurs abundantly in the vicinity of Woods Hole, Mass., and gametes may be secured throughout the summer months. Shedding takes place immediately following the removal of the animals from their tubes.

2. The early development is described from shedding to early cleavage and a comparison made with *Sabellaria alveolata* as described by Fauré-Fremiet. In most respects the early developmental phenomena of these two species are strikingly similar.

3. After shedding the eggs become spherical, the vitelline membrane elevates, leaving fine protoplasmic processes between membrane and egg cortex, and the contents of the germinal vesicle form a clear area at the animal pole. Visible polarity appears during pre-maturation which ceases at the metaphase of the first maturation spindle.

4. It is said that fertilization occurs normally during May and June but a second and shorter time has been found during the early part of August. During July a high percentage of fertilization may be secured with slightly alkaline sea water (8.6).

5. The highest percentage of cleavage is obtained when the gametes are shed together. Eggs may be fertilized after seven hours, but the fertilizing capacity of the spermatozoa is considerably shorter (about one hour).

6. The fertilization phenomenon is described. The protoplasmic strands serve to transmit the stimulus of sperm contact. No fertilization membrane appears and the first indication that a spermatozoön has entered is the withdrawal of the processes.

7. The fertilization cone is enormous and changes shape. Spermatozoa may enter at any point on the periphery but more frequently in the animal hemisphere. The polar bodies are large and the first one frequently divides.

8. Various factors such as increase of pH, shaking, and standing are sufficient to disturb the equilibrium of the pre-maturation spindle with the resultant formation of one or two polar bodies. This is followed by lobular fragmentation and no swimming embryos develop. Old unfertilized eggs stratify into three zones.

9. Plane of first cleavage bears no fixed relationship to sperm entrance point and passes to one side of pole as determined by the position of the polar bodies. The polar bodies lie on the smaller blastomere while the larger one contains the polar lobe material.

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CATALASE AND OXIDATIVE PROCESSES IN ANIMAL TISSUES AS POSSIBLE FACTORS IN ADAPTATION

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In a previous report (1930) upon the respiratory exchange and catalase in tissues of lamellibranchs, the author pointed out a relationship between the habitat distribution of several species and the relative abundance of their muscle catalase. To these have been added new data for the same and for other species on our southern Atlantic coast, making twelve altogether and representing diverse conditions of environment. The above experiments were performed at the U. S. Bureau of Fisheries, Beaufort, North Carolina, during several summer seasons. A second series of determinations was carried out at the Bermuda Biological Station in the summer of 1931, using four other species.

The data from the Carolina and Bermuda experiments, summarized in Tables I and III, are treated separately because the species are distinct and on account of climatic differences in the two regions. For, although the temperature of the water is practically the same in the summer, the annual variation is greater on the Carolina coast than in Bermuda. The fauna in the latter region is sub-tropical in character.

The Bermuda species of bivalve mollusks used in this study differ in certain respects from those on the mainland. They are attached firmly to the substratum by means of a byssus (*Mytilus, Arca*), or with one valve of the shell cemented to rock or piling (*Chama, Spondylus*)—and all are non-burrowing. A majority of the Carolina species burrow in sand or mud. Of the others, *Modiolus* and *Arca ponderosa* have a byssal attachment and live exposed in varying degrees; *Pecten* is free-swimming; *Ostrea* is attached securely to solid objects, freely exposed. In these species the figures for oxygen consumption are higher and more diverse than in the Bermuda forms. Two varieties of *Arca* observed in Bermuda were entirely lacking in the red blood pigment which characterizes members of the genus on our coast.[1] The tropical conditions seem to be conducive to uniformity in the physiological activities of the species.

In comparing results for muscle catalase and respiration in different species, it is assumed that they are representative of whole animals, since muscle makes up the bulk of the tissue in these mollusks. The adductor muscles, moreover, are the organs most directly involved in sustained closure of the shell, in which the greatest economy in oxygen metabolism is required. This point will be discussed later.

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[1] Svedberg (1933) finds that the respiratory protein of *Arca pexata* has the same properties (molecular weight, etc.) as that in other invertebrates. This he calls erythrocruorin, as distinct from the hemoglobin of vertebrates.

METHODS

The oxygen consumption of muscles was measured with respirometers of the Thunberg type. Many of the data for certain species, which have been previously published (1930), are incorporated in this paper. Hence the same procedure, of suspending blocks of tissue in air, was followed in later work, in order to obtain results comparable with the others. Single portions of adductor muscle (and of pedal retractor in *Pinna*) were used, thus allowing individual comparisons to be made, except in the case of *Mytilus exustus,* whose small size necessitated the use of muscles from several specimens, bound together as one. The average amount of tissue represented for each species was nearly the same (between 271 and 304 mg.), although the diversity in individual experiments was somewhat greater. The results given were directly calculated as cubic millimeters of oxygen used per gram of tissue per hour (at 27.5° C., not corrected for barometric pressure). Readings were from one to two hours in duration, after allowing one hour for equilibrium to become established. While these may not represent maximal rates of oxygen consumption under conditions favoring perfect diffusion of oxygen into tissues, they are quite adequate for showing the outstanding differences between species. The oxygen taken up by thin sections of muscle, placed in sea water saturated with oxygen and shaken continuously, was determined in certain species, and similar differences in rate of respiration were found as by the above method. Thus, the following figures were obtained: for *Ostrea virginica* (11 experiments), 48.6 cu. mm.; *Venus mercenaria* (6), 33.8 cu. mm.; *V. campechiensis* (4), 28.9 cu. mm.; *Dosinia discus* (16), 62.6 cu. mm.; *Cytherea gigantea* (7), 28.3 cu. mm. Inadequate diffusion of oxygen into the tissue seems to be unimportant as a factor limiting the rate of oxygen consumption, except in *Dosinia,* which has the highest rate of all. Furthermore, the wet weight of the tissue, which forms the basis of these measurements, has been found to be sufficiently reliable for the comparison of results in different species or in types of muscle tissue. All experiments were carried out at 27.5° (± 0.3°), which was approximately the mean temperature of the water in which the animals lived during the greater part of the summer.

Catalase determinations were made at the end of every respiration experiment, on the same tissues. The depletion of catalase in unsectioned muscle, during the four-hour period which elapsed from the time of excision, did not exceed about 10 per cent. It was often much less in species with low muscle catalase content. Hence, the figures in the tables representing catalase and the ratio of oxidation rate to catalase show less extreme differences for species at opposite ends of a series than actually exist, since no correction for depletion was made.

The estimation of catalase was according to the method described in 1930. In all experiments in Bermuda, however, and in those done at Beaufort subsequently, the amounts of extract and of peroxide were kept constant. Thus 20 cc. of a 1 per cent tissue suspension and 2 cc. of 3 per cent hydrogen-peroxide solution were used, giving a 0.27 per cent concentration of H_2O_2 after mixing. The oxygen evolved in 5 minutes was determined at 27°. Owing to the small amount of muscle tissue available in a single specimen, it was found to be impracticable to adjust the concentration of extract so as to decompose a definite percentage of H_2O_2 in a given time. In order to limit decomposition of the reagent to about half, however, a muscle extract of 0.5 per cent concentration was used in all experiments with *Mytilus,* in 13 with *Ostrea,* and in 6 with *Pinna.* Extracts (1.0 per cent) of *Ostrea* and *Pinna* adductors which gave about half decomposition of the peroxide, when diluted to one-half and to one-fourth strength, liberated almost proportionate amounts of oxygen. There was some variation in the activity of these extracts in different concentrations: and on standing, the catalase sometimes increased, then decreased. The average error from these sources is within the limits of ± 5 per cent. In 10 determinations in *Ostrea* and 2 in *Pinna,* results were obtained by extrapolating from 2-minute readings (using 1 per cent suspensions), and correcting for excess of catalase in relation to H_2O_2. This was unnecessary when the 5-minute readings obtained with the stronger extracts were sufficiently low.

RESULTS

The Carolina species, as listed in Table I, are arranged in general according to their ability to withstand unfavorable conditions, such as exist in poorly aerated aquaria, or under partly anaerobic circumstances in their natural environment. Whether this arrangement conforms to their distribution according to habitat is not always clear, except for the two or three species at each end of the series, because of overlapping. The diverse conditions of environment and of morphology also complicate the situation. *Ostrea* and *Modiolus* are the only non-burrowing species which are periodically exposed at low tides, at which times the shell remains closed and the animal is subjected to the heat of the sun.[2] *Modiolus* is somewhat less affected by exposure, because it lives on grassy tidal flats, where it may be shaded or partly covered with mud. The other non-burrowing species, *Arca* and *Pecten,* are seldom found above low water level, and show less viability in the laboratory, hence belong lower down in the series. The position of the remaining species, however, must be decided mainly according to their proximity to marshes or to open beaches; their relative size, and activity; the extent to which the animal can withdraw into its shell, and the depth to which it burrows. *Pinna* is a large mussel (up to 30 cm. in length) which inhabits scallop beds, or mud flats on which it is sometimes exposed at low tide. Since the posterior end of the shell protrudes a few centimeters and does not always close tightly, the water within the mantle chamber may be partly lost if the animal is exposed. *Tagelus,* a form having long external siphons, lives in a burrow almost a foot beneath the surface, on sandy mud flats and beaches. Despite the

TABLE I

North Carolina Series

Species	No. of individuals	No. of experiments	Weight of muscle	Oxygen used per g./hr.	Catalase	Ratio $\frac{\text{oxygen}}{\text{catalase}}$, and S.D.
			mg.	*cu. mm.*		
Ostrea virginica Gmelin	24	29	271	46.4	68.4	0.78 ± 0.28
Modiolus demissus Dillwyn	10	13	291	32.7	31.7	1.00 ± 0.25
Pinna muricata (Linnaeus	12	22	278	40.5	71.3	0.83 ± 0.52
Tagelus gibbus (Spengler)	10	12	280	47.1	33.2	1.43 ± 0.37
Venus mercenaria Linnaeus	30	60	290	38.5	19.8	2.16 ± 0.80
Venus campechiensis Gmelin	10	12	289	35.6	11.5	4.33 ± 1.83
Arca ponderosa Say	10	18	285	29.1	14.1	2.22 ± 0.86
Pecten irradians Lamarck	14	14 striated	281	78.5	19.1	4.53 ± 1.92
		14 plain	275	39.9	31.3	1.32 ± 0.31
Cytherea gigantea Gmelin	10	16	280	28.0	9.5	2.98 ± 0.67
Dosinia discus Reeve	11	15	281	51.4	16.1	3.18 ± 0.65
Cardium magnum Born	8	11	281	30.7	9.5	3.53 ± 1.22
Mactra solidissima, Var. similis Say	6	10	277	41.8	9.5	4.86 ± 1.73

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[2] Although *Ostrea* occurs also in deeper water in certain localities, it is almost restricted to the intertidal zone in Beaufort Harbor. The factors which determine vertical distribution in the oyster are discussed by Prytherch, 1934.

tendency for the shell to gape widely in specimens placed in open aquaria, this species seems to bear mutilation better than *Venus mercenaria*, in which removal of a shell segment without visibly injuring the mantle is usually fatal. In this respect *Tagelus* resembles *Ostrea* and *Pinna*. Six species remain to be considered: they are all closed-shell forms (with retractile siphons), and burrow just beneath the surface of the sand, or, in the case of *Dosinia,* to a depth of about one foot. The species of *Venus* occur on sandy beaches, and on mud bottoms near the marshes as well. Only an occasional specimen of *Cytherea, Dosinia,* or *Cardium* was ever found by the author near the Fisheries Laboratory, and none of *Mactra*. These last four could be collected, however, on a sandy reef (Bird Island) halfway between the laboratory and the entrance to the harbor. The outer part of this shoal, which was out of water only at low tide, was exposed to the action of waves and currents from the open ocean through the harbor inlet. *Cytherea* was usually found well up on the shoal—but not beyond eight or ten inches elevation—in coarse sand or gravel; *Dosinia* just at low water line; *Cardium* also at low water line, but more often under water; *Mactra* only beneath the surface at mean low tide. Shells of *Dosinia* and *Cardium* and of *Mactra solidissima* Chemnitz were plentiful around the inlet and on the ocean beach.

As may be seen from the figures (Table I), much diversity obtains as regards the catalase content of the muscles, whereas the oxygen consumption is more constant. Those species which are intertidal, or which inhabit mud flats, usually have the largest amounts of muscle catalase; those which live almost exclusively along sandy beaches and are not exposed to the air at low tide have least. There are quantitative exceptions to this rule, however, which would suggest that other factors influence distribution in these forms. Thus, in *Dosinia,* the figure for catalase is almost twice as great as that for other species inhabiting a similar environment (*Cardium, Mactra*). In *Modiolus* the amount of catalase is less than one might expect for a species which lives partly exposed between tide levels. May it not be that the magnitude of respiration also affects adjustment to environment?

That the two factors, oxygen consumption and catalase in the tissues, are important in adaptive distribution is indicated when we take into account the ratio of the two. Dividing the first figure by the second, for each individual of a species, the mean numerical quotient (last column of table) gives us a fair indication of the distribution of that species. In the case of *Dosinia,* this quotient serves to place it with the other species found in the same habitat (see above). Likewise, in

Modiolus, the ratio (oxygen: catalase) assigns it to a position higher in the series than the figure for catalase would do.

A comparison of the two species of *Venus* reveals a difference in muscle catalase which is rather striking in view of their similarity and somewhat parallel distribution. Some specimens, in fact, are difficult to identify, and are possibly hybrids. In one individual of *V. campechiensis*, the reading for catalase was found to be 41, although it was less than 14 in each of the others; a fact which also suggests intercrossing, with segregation of physiological characters. The figure for the oxygen/catalase quotient, too, is somewhat higher than one would expect in this species, in view of its wide local distribution. *Venus mercenaria* occurs along sandy beaches, on mud flats, or even in marsh grass areas; it can tolerate semi-stagnant conditions (in terrapin pounds, etc.), and can live on parts of the beach which are not ordinarily covered by water more than one-third of the time. *V. campechiensis,* according to the author's experience, is found in sand, with *Dosinia* and *Cardium;* or on mud shoals along the river channels (near the laboratory), at low water line and out at a considerable depth. It seldom occurs more than a few inches above low water level even on the sandy beaches. These differences—in tolerance, and in vertical distribution—help to explain the divergent results obtained in the two species for catalase and O/C quotient.

TABLE II

Age Differences in Venus mercenaria: Oxygen Consumption and Catalase

Age group	Number of specimens	Oxygen consumption	Catalase	Ratio $\frac{\text{oxygen}}{\text{catalase}}$, and S.D.
Young clams	16	44.7	17.5	2.68 ± 0.59
Old clams	14	31.4	22.6	1.56 ± 0.53

The relation of *Pecten* to the other species in this series is not clear, because it is highly specialized and lives in no fixed habitat. The main portion of the adductor muscle—comprising more than three-fourths of the whole—consists of cross-striated fibers, which would doubtless play a predominant rôle in the metabolism of the animal. This being true, the scallop should perhaps be placed in a class with the active species (*e.g. Cardium*) characterized by low catalase.

In general, the species in this series which display great variability in shell form etc. are widely distributed and exhibit much diversity in the experimental results (*Ostrea, Pinna, Venus mercenaria, V. campechiensis*); those which show least variability are often restricted to particular regions or habitats, and give more uniform results in the determinations (*Dosinia, Cytherea, Modiolus*). The relative variation in experimental data is represented fairly well for each species by the standard deviation of the oxygen/catalase quotient in the last column of Table I. In *Pinna*, and to a less extent in *Ostrea*, this variability affects the catalase rather than the oxidation rate, but in the other species it is about equally shared by the two. Special conditions may have influenced the results in certain species, as, for example, the difficulty of finding more than one specimen at a time, and of keeping it in a healthy state until used (*Cardium, Mactra*); the specialized morphology of the adductor in Pecten; or the individual age differences within a species.

The age factor plays a minor rôle in this comparative study of species, because mature specimens were usually required for the experiments. In Venus mercenaria [3] the data for young and old individuals are about equally represented, so that the influence of age on oxygen consumption and catalase can be seen by comparing the averages in the two age groups (Table II); just as in different species, the lower oxygen/catalase quotient is associated with the greater power of survival displayed by mature clams, as compared with young ones, when kept out of water.

The degree of muscular activity exhibited in species of this series is inversely related to the amount of catalase in their muscles; and, on comparing the two diverse types of muscle making up the adductor in *Pecten*, a similar correlation is seen. There is distinctly less catalase in the rapidly contracting, cross-striated muscle of the scallop than in the slow, non-striated part. The rate of oxygen consumption, as determined in the striated muscle after excision, is perhaps higher than the resting value because of a greater responsiveness to stimulation as compared with the smooth muscle. On comparing different species, it may also be said that in the actively burrowing forms there is least muscle catalase (*Cytherea, Cardium, Mactra*); in the more sluggish species which have a foot adapted for digging, there is usually more (*Tagelus, Venus mercenaria, Dosinia*). Although *Dosinia* is found in the same habitat with the first three (active) species, it seems incapable of performing the sudden movements which characterize these, such as leaping in an aquarium.

The four species of Bermuda bivalves (Table III), while morphologically distinct, live in a rather uniform physical environment and hence can be compared directly from the standpoint of their vertical distribution. The average oxygen consumption is nearly identical in each, so that the differences in catalase (and in the oxygen/catalase quotient) are not conditional upon this factor. *Mytilus exustus* is the only intertidal species, occurring at levels up to a foot or more above low tide mark. Its scarcity below this level may be partly due to the depradations of its natural enemies. *Arca occidentalis* and *Chama macrophylla* are frequently found exposed just above low tide, but are much more abundant below this level. *Spondylus americanus* lives in comparatively deep water, and is seldom collected in water less than one foot deep in the inner passages, or in less than half this depth on the outer ocean shore. The author has never seen but one specimen living, under natural conditions, above low water mark—and then at an exceptionally low tide, on the shaded side of a bridge pile, where the current was swift.

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[3] In a previous tabulation of results for *Venus mercenaria* (1930), there were included data for the closely related *V. campechiensis*—Table 2, under August 18 (2) and August 19 (2)—before these species were clearly recognized as distinct. The two specimens were later identified by their shells, and the data are included in this paper under *V. campechiensis*. This change does not materiallly affect the conclusions previously drawn.

TABLE III

Bermuda Series

Species, or variety		No. of individuals	No. of experiments	Weight of muscle	Oxygen used per g./hr.	Catalase	Ratio $\frac{\text{oxygen,}}{\text{catalase}}$ and S.D.
				mg.	*cu. mm.*		
Mytilus exustus Linnaeus...		44	8	288	23.7	44.3	0.54 ± 0.12*
Arca occidentalis Philippi...		10	20	283	24.4	23.8	1.08 ± 0.32
Chama macrophylla Chemnitz	"brown" variety	10	17	285	24.2	24.1	1.24 ± 0.61
	"red" variety	6	11	277	22.5	10.4	2.18 ± 0.33
Spondylus americanus Lamarck...............		10	20	304	25.8	9.2	3.01 ± 1.00

* Based upon the number of experiments (not individuals, as in the other species).

Certain differences in horizontal distribution may be worth noting, as a basis for comparing *Arca* and *Chama* as to their habitats; likewise for the varieties of *Chama macrophylla*. All of the species here represented could be found in Ferry Reach, a rather shallow inner passage, near which the Bermuda Biological Station is located. This communicates with the ocean at a distance of more than a mile, and in the other direction with Castle Harbour. On the outer ocean shore specimens were less numerous, and smaller, doubtless owing to a less abundant food supply. *Mytilus* was collected only in Ferry Reach and its inlets. One of these inlets, serving as an anchorage for the laboratory boats, terminates in a shallow mangrove swamp with a muddy bottom. At low tide many specimens of *Mytilus* were found above water on rocks a short distance below this mangrove area, and a few of *Arca* at the water line. *Chama* was absent here, but a few specimens were collected farther down the inlet, below the boathouse, where the water is deeper.

Chama macrophylla occurs in Bermuda in abundance, and displays much diversity in form and coloration. The border of the mantle may be deeply pigmented with brown, or various shades of grayish-buff, especially around the siphons. There are evidently different color phases represented in this species, or at least intergrading color combinations; and two of these appear to be distinct. In one the mantle is brown or buff, and the inner shell-surface near the border is of some shade of lavender. In the other variety the siphons are bright red in color, the inner margin of the shell some shade of chestnut.

While investigating the respiration rate and catalase in tissues of this species, it was noticed that, whereas the first was of about equal magnitude in the two color varieties, the readings for catalase were different—although overlapping somewhat (Table III). Since the average result in the red variety was found to be less than half that in the brown variety, it was thought that catalase might be associated with a difference in vertical or regional distribution. No obvious difference in the vertical distribution of the red and brown varieties was observed, but on comparing their relative abundance in distinct localities an interesting result appeared. In a collection of this species made in Ferry Reach, mainly from the Swing Bridge piers, in a total of 155 individuals, 18 (or 11.6 per cent) were of the red variety, 137 (or 88.4 per cent) of the brown variety. A similar count of 43 specimens collected on the outer (north) shore revealed 17 (or 39.5 per cent) of the red, and 26 (or 60.4 per cent) of the brown variety. Whether this proportional difference in distribution represents a partial segregation of the two varieties, based upon their known characteristics, is a matter for conjecture. It is unlikely that color variations would have adaptive significance in relation to such similar habitats. But different degrees of catalase activity of the tissues, perhaps by influencing their underlying oxidative capacity, might determine the relative fitness of individuals for one or the other kind of environment. Animals which live in the open ocean as compared with the inside water passages, are less subjected to daily fluctuations in temperature, and they are exposed to continuous wave action, which, however slight, is a factor of importance. The red variety of *Chama macrophylla,* having less catalase in its muscle tissue than the brown one, is the form which one would expect to find in relatively greater numbers on the outer shore, as was found to be the case in comparing Beaufort species.

Spondylus resembles the scallop (*Pecten*) most closely of all the species studied, except that it is sessile. The two genera are very similar in the physiology and differentiation of their adductor muscles, as well as in gross morphology. In the quick-contracting muscle of *Spondylus gæderopus,* as described by Marceau (1909), the striations are apparently transverse or "en chevrons"; hence they correspond with the cross striations of these fibers in the scallop rather than the oblique striations in the homologous muscle of the oyster and other forms. In *Pecten* the striated muscle was found to have less catalase than the non-striated portion, in every specimen. This is also true for seven individuals out of ten in the case of *Spondylus,* although the differences are small.

DISCUSSION

In the main, the facts in regard to the amount of muscle catalase in different species, in relation to habitat, seem clear; but the interpretation of their significance rests upon our imperfect knowledge of the mechanism of cell oxidations. A detailed comparison of species is not always possible, owing to the diversity of forms studied, the complexity of their environment, and the many sources of experimental error. It may be said, however, that the same order of relationship obtains for the species in the two regions. That is, there is a positive correlation, in each series, between muscle catalase and the ability of species to withstand anoxic conditions. Because of individual exceptions, the ratio between oxygen consumption rate and catalase (O/C quotient, p. 117) seems to represent more accurately this relationship. As suggested previously (1930), catalase possibly plays a protective rôle by regulating physiological oxidation in the muscles of bivalve mollusks, and thus determines the extent to which these are adapted to anærobic life. The presence of much catalase, in the muscles of species which are forced to keep the shell closed for long periods of time, might make for greater economy in the maintenance of tonus, in which only a small increase in metabolism is required. In active species its presence in excess would interfere with sudden demands upon the metabolism of the muscles in rapid, repeated contractions. To the objection that catalase is lacking, or present only in small amounts, in anærobic organisms, it may be said that mollusks are not typical anærobic forms such as derive energy primarily by fermentation.

The function of catalase is considered to be the decomposition of hydrogen-peroxide formed in tissues, thus preventing its accumulation in amounts toxic to the oxidizing enzymes. Since molecular (inactive) oxygen appears in the reaction, it does not promote oxidation directly, but is still available for oxidases to act upon. That catalase has such a protective function in relation to xanthine-oxidase was demonstrated by Dixon (1925). Moreover, a large amount of catalase, by destroying H_2O_2, retards oxidation with peroxidase, but does not entirely prevent it, since peroxidase reacts with a much lower concentration of peroxide than does catalase (Thurlow, 1925). One can thus explain the rôle of catalase in animals under conditions of oxygen deficiency, by its conserving action on metabolism.

The extension of this investigation to other groups of organisms would doubtless help to clear up many points in regard to their environmental segregation. One such case is that of the occurrence of *Tellina tenuis* between tide marks in the Clyde estuary (Elmhirst, 1932). The greater relative abundance of large specimens at high tide level, and of small ones near low water in an equal area of sand, is understandable on the basis of the greater powers of survival of old individuals out of water, as postulated for *Venus mercenaria* (Table II, and context).

Summary

The catalase and oxygen consumption of the muscles are compared in twelve species of lamellibranchs in North Carolina, and in four in Bermuda. In each region, those species which are intertidal, or which would be subject to partly anærobic conditions, are characterized by larger amounts of muscle catalase than those which live continuously submerged. There is a proportionality between rate of respiration and catalase in muscle for species in a given habitat. The significance of these results is discussed in relation to current views regarding physiological oxidation.

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A METHOD FOR THE DETERMINATION OF AMMONIA IN WATER AND AIR

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In the course of a study of the organic material present in solution in sea water, it became necessary to measure also the quantity of nitrogen present as ammonia, and, since the method worked out for this purpose appears to be of wider applicability, especially in oceanography, I propose to describe the method finally arrived at in some detail. It can, with a little practice, be carried out fairly easily and very rapidly even on board ship, a condition which appears to be absolutely essential, since no treatment has been discovered so far which will keep the ammonia of sea water samples at the original level.

The principle of the method is to make a sample of water containing from 0.05 to $2\,\gamma$ (gamma $= 0.001$ mg.) ammonia nitrogen (generally 20 ml.) just alkaline, to distill off in a partial vacuum the ammonia together with about 5 ml. of the water into a small volume of weak acid in which the ammonia can be titrated with naphthyl red according to the method of Teorell (1932).

THE DISTILLATION

The following reagents are required:

1. Re-distilled ammonia-free water is prepared from tap water or distilled water containing about 1.5 grams permanganate and 0.1 ml. sulphuric acid per liter by distillation from a Pyrex flask of 2–3 liters capacity. A very effective trap for catching drops is essential and the permanganate is useful mainly because its presence in the trap shows how far drops are carried. About 5 grams of pumice are added to insure quiet boiling. The middle portion of the distillate is taken up in 1-liter glass-stoppered Pyrex bottles and, as a measure of safety, a current of CO_2- and NH_3-free air is blown into the bottle during the distillation. Water for the day's use is poured from the storage bottle into a glass-stoppered bottle of suitable size. This water is used for rinsing and for the preparation of certain further reagents and control samples.

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[1] Contribution No. 28.

2. About .25N sodium hydroxide, prepared by dissolving 1 gram of NaOH in 100 ml. NH_3-free water, is used for bringing the water samples to a hydrogen-ion concentration between pH 9 and 11. A sample of the water (20 ml. or a suitable fraction) is titrated in a white porcelain basin with the alkali from a 1- or 2-ml. burette, using cresolphthalein as an indicator. The corresponding amount of alkali or a little more is added to the water sample just before distillation. I have found that even an excess of 50 per cent alkali does not liberate any excess ammonia from the waters so far tested.

3. The ammonia distilled off is taken up in 1 ml. hydrobromic acid about N/100 prepared by adding 0.2 ml. 25 per cent HBr to 133 ml. ammonia-free water. This solution must remain NH_3-free, and if the air in the laboratory shows traces of ammonia it should be protected from contact. Neither the concentration nor the quantity of hydrobromic acid measured off need be determined with any particular accuracy.

The distillation apparatus is constructed upon the general lines laid down by Parnas and Wagner (1921), but certain modifications have been introduced, the most important of which is the use of air at a reduced pressure instead of steam for driving off the ammonia. The distillation flask (Fig. 1, 1) is of Pyrex glass and holds at least 50 ml. in the bulb. It is provided with a standard ground joint of 14–11 mm. Slightly alkaline glycerine is used to lubricate this joint. At least two and preferably three of these flasks should be available for rapid serial determinations.

The connecting piece (2) must also be Pyrex, but the rest of the apparatus can be made from ordinary soda glass. The final 2–3 cm. of the tube (3) should be capillary tubing of 0.5–0.6 mm. diameter constricted at the tip to about 0.1 mm. The part below the condenser can be conveniently constructed from a gas-washing bottle. The bottom is replaced by a rubber stopper through which a glass rod can be pushed up and down to place the collecting bottle (4) at just the right height. For serial determinations a suitable number of these bottles should be available. A metal ring (5) is sealed on to the inside of the bottle with DeKhotinsky cement to prevent the stopper from being sucked in by the vacuum. The stopper and glass rod are kept well lubricated with alkaline glycerine. The 3-way tap (6) connects this part of the apparatus with a vacuum or effective filter pump. Ammonia-free air is admitted to the apparatus through the sulphuric acid bottles (7) and (8). The tube (9) is between 60 and 100 cm. in length and about 0.1 mm. bore and is permanently open. It should provide just enough air for driving over the ammonia when the vacuum is on. The tube (10) is wider. The screw clip (11) is opened only when the distillation is finished and the pressure raised to that of the atmosphere. When doing a series of distillations, I have always found it necessary to begin with one or more blanks to get rid of traces of NH_3 which will collect even in the closed apparatus. The collecting bottle is charged with 1 ml. N/100 HBr and put in position so that the tip of the tube (3) will just dip into the fluid when the vacuum is put on. About 20 ml. ammonia-free water is put into a distillation flask with a single drop of N/4 NaOH. The evacuation is started as soon as the distillation flask is put on and the burners (11) and (12) ignited. The burner (11) is regulated so as to produce a suitable rate of distillation, usually about 1 ml. per minute. The burner (12) prevents condensation in (2). The bottle (4) is lowered gradually as distillation proceeds so as to minimize splashing, and before it is finished the tube (3) should be clear of the fluid. After distillation of about 5 ml., the gas is turned off, the tap (6) turned so as to shut off the apparatus from the pump without opening it to the atmosphere, and the screw clip (11) opened so as to admit a fairly rapid current of air. It is important to have the gas turned off between determinations as it usually contains appreciable amounts of ammonia. Finally the bottle (4) is taken out and immediately stoppered.

During the distillation of one sample there is time to prepare a fresh collecting bottle and the next sample for distillation. The alkali is added to the latter in the interval during which the pressure in the apparatus is being brought back to the atmospheric level. Following this procedure five determinations can be made in one hour.

THE AMMONIA TITRATION

This process has been worked out according to the excellent method introduced by Teorell (1932). Only minor alterations have been made to deal with quantities even smaller than his. The principle of the method is to oxidize the ammonia with a surplus of sodium hypobromite in alkaline solution. After acidification the excess hypobromite is titrated with an acid solution of naphthyl red which becomes decolorized so long as NaOBr is still present.

The necessary reagents are:

1. Sodium hypobromite about n/1,000, made up by dilution from

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FIG. 1. Apparatus for the determination of pre-formed ammonia in water and air.

(1) Distillation flask. (2) Connecting head. (3) Condenser. (4) Receiver. (5) Receiver-holder. (6) Three-way tap connecting with vacuum. (7) and (8) Sulphuric acid wash bottles. (9) Fine capillary for constant admission of air. (10) Clamp for returning to atmospheric pressure. (12) *-a-*, *-b-*, micro-burners. (13) Bottle with syringe-pipette for hypobromite solution. (14) Hydrobromite acid. (15) Absorber for the determination of ammonia in air.

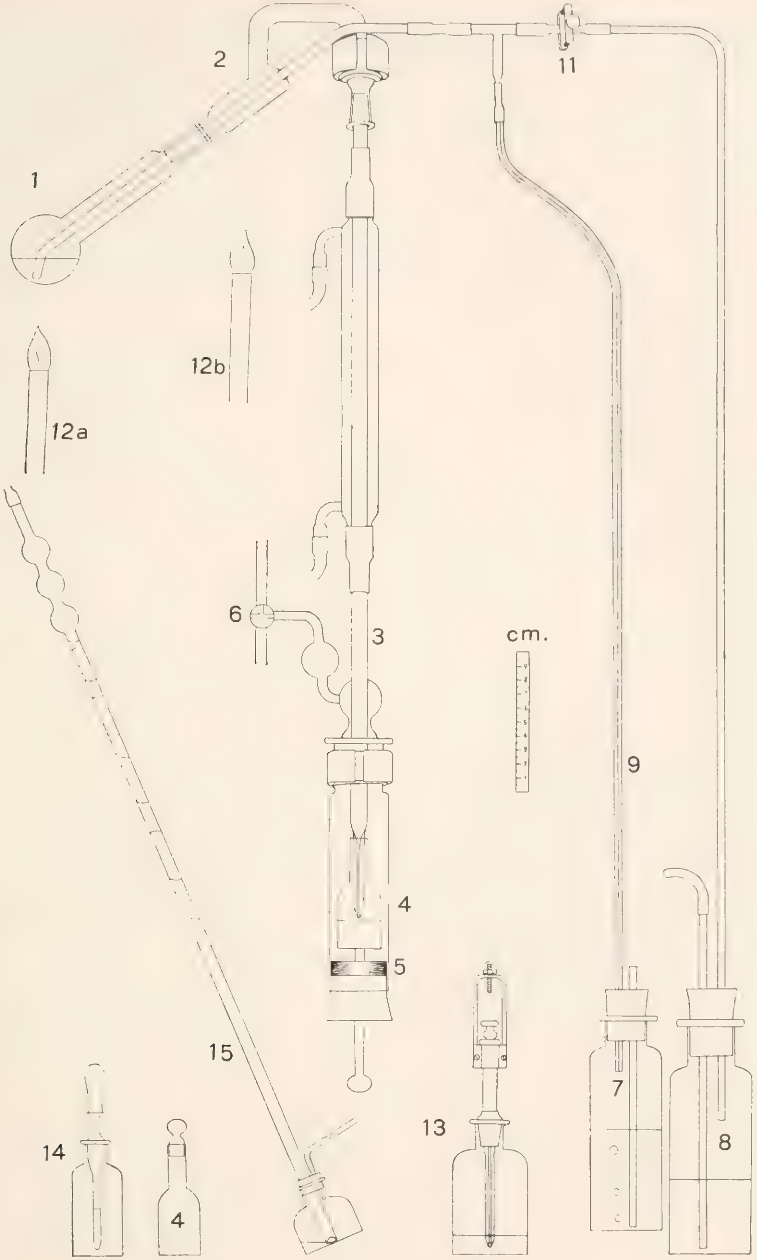
2
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FIG. 1

n/10 stock solution (2.5 grams NaOH, 1.25 ml. fluid bromine in water to 500 ml.). The reaction between NaOBr and NH_3 must take place at a pH of 8.5–9 and the n/1,000 sodium hypobromite is therefore made sufficiently alkaline with 2n Na_2CO_3 to give this pH when about 1 ml. is added to 1 ml. of n/100 HBr. I have found 5 ml. of 2N Na_2CO_3 per 500 ml. suitable.

The stock solution will remain constant indefinitely when kept at a low temperature. The dilution, which must be kept in a dark brown bottle, is not quite constant, but can be used for weeks. I have found it convenient to transfer the quantity for each day's use into a smaller brown bottle and to keep the stock in complete darkness.

2. Hydrobromic acid about 30 cc. 25 per cent diluted with ammonia-free water to 200 cc.

3. Naphthyl red n/2,000 made up by mixing in the order indicated 30 cc. glacial acetic acid, 3.75 cc. 89 per cent H_3PO_4, 1.9 cc. 25 per cent HBr, 10.5 cc. of a freshly prepared 0.1 per cent solution of naphthyl red in water, water to 500 cc.

This solution keeps well, but a small amount of a brown precipitate which interferes with the titration is often formed, and the best plan is to filter off each day the quantity necessary for the day's use.

4. Ammonium sulphate. The stock solution contains 0.4716 gram $(NH_4)_2SO_4$ in 1 liter. This is kept protected against evaporation.

One milliliter of this diluted to 100 ml. with NH_3-free water will contain 1 γ nitrogen per milliliter.

It is convenient to measure off the hypobromite for each titration with a syringe pipette mounted in the bottle as shown in Fig. 1 (13). The volume of the pipette need not be just 1 ml., but is regulated so as to correspond to something between 1.8 and 2.0 ml. naphthyl red. The hydrobromic acid I keep in a small bottle (14) with a ground-in rubber-capped pipette which will deliver approximately 0.2 ml.

The titration is made from a 2-ml. burette divided in 200 parts. It is necessary to do each day 2 to 4 blanks and 2 to 4 titrations of a known amount of ammonia. The results depend to some extent upon the temperature, and when the room temperature undergoes considerable variation more constant results are obtained if the solutions to be titrated are brought to an approximately constant temperature in a water basin.

To do a blank, measure off in the titrating bottle 1 ml. n/100 HBr, add about 5 ml. NH_3-free water, bring to the desired temperature, add hypobromite and shake gently; fill up the burette with naphthyl red to the zero point. This takes about the half minute required for the reaction between hypobromite and ammonia to become complete. In order to secure uniform results constant timing should be adhered to. To the sample is now added 0.2 ml. 4 per cent hydrobromic acid; it is again shaken up and titrated from the burette to a just visible rose color. It is advisable to use a comparison bottle with water and just enough naphthyl red to produce this color. The titration must not be made too rapidly, but should take at least 1 minute. For the determination of the NH_3 equivalent of the naphthyl red, 1 or 2 ml. of the ammonium sulphate solution is added to the n/100 HBr and enough NH_3-free water to bring the volume to 5–6 ml. The mixture is treated as above and titrated. To test the absorption of NH_3 during distillation, ammonium sulphate is added to 20 ml. water and distilled off.

Example:

	ml. naphthyl red solution
Blanks titrated directly	1.96, 1.95
Blanks distilled	1.95, 1.95
Ammonium sulphate (2 γ N) directly	0.89
Ammonium sulphate (2 γ N) distilled	0.89

2 γ N = 1.06 ml. naphthyl red solution

For the determination of ammonia in fluids where less than 1 γ is regularly to be expected, it may be worthwhile to reduce the volume of hypobromite to .5 ml. The accuracy to be obtained in the single determinations of a series is of the order 0.04 γ N which corresponds by the distillation of 20 ml. to ± 2 mg. ammonia nitrogen per cubic meter.

The Determination of Ammonia in Air

An apparatus like the one shown (Fig. 1, 15) is suitable. The glass tube is 6 mm. in internal diameter and about 50 cm. long up to the lower of the three bulbs. The top is connected to a water bottle of suitable capacity (4 to 10 liters) from which the water is allowed to run out at a rate of 4 liters per hour or less. The bottle is charged with 1 ml. N/100 HBr and 4–6 ml. NH_3-free water and the whole is placed in a slanting position so that the absorbing fluid moves fairly regularly right up to the bulbs, thus providing a constantly renewed absorbing surface of about 100 $cm.^2$ Titration is performed as above in the absorbing bottle. The air in a laboratory for ammonia determination should contain less than 0.1 γ N per liter.

Summary

A method and apparatus for the determination of ammonia in water are described. The ammonia is carried over by a simple vacuum distillation into hydrobromic acid in which it is determined by the naphthyl red titration of added hypobromite.

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METHODS FOR THE DETERMINATION OF DISSOLVED ORGANIC CARBON AND NITROGEN IN SEA WATER

AUGUST KROGH AND ANCEL KEYS

(*From the Laboratory of Zoöphysiology, Copenhagen University, and the Woods Hole Oceanographic Institution*[1])

Introduction

Certain problems in the nutrition of marine animals and in the general economy of organic life in the sea involve the determination of organic matter present in solution in the water. The methods worked out by the senior writer for the determination of dissolved organic matter in fresh waters are of no avail. In these methods (Krogh, 1930) the organic material is concentrated by evaporation of a large sample of the water and the organic material determined by combustion of the dry residue, but the sea water salts, present in quantities several thousand times as large as the organic material, make any attempt in this direction futile. All we can hope to accomplish by means of the resources at present available are determinations—more or less approximate—of the nitrogen and carbon present in organic combination, and these simplified analytical problems are what we have endeavored to solve.

Assuming according to the scanty and not especially convincing estimates to be found in the literature (Atkins, 1922; Brandt, 1916–20; Gran and Rund, 1926; Harvey, 1925; Natterer, 1892–1900; Vernon, 1898) that we should have to deal with quantities of at most a few milligrams per liter of water, it has been our endeavor to work out microchemical methods of handling water samples of small volume. Large samples are in many cases not available, and, more important, the necessary time and expense in preparing large samples for analysis would be a serious drawback.

The Determination of Organic Nitrogen

A large number of trials were made with micro Kjeldahl methods, but they all failed. The quantity of sulphuric acid necessary for the Kjeldahl treatment becomes relatively very large on account of the salts, and it was not found possible to obtain or prepare sulphuric acid of sufficient purity. All samples examined would, after the Kjeldahl

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[1] Contribution No. 47.

heating in ammonia-free air, give off a variable amount of ammonia when distilled after addition of excess sodium hydroxide. The sulphuric acid was treated in various ways to get rid of this "organic" nitrogen and in the final procedure evaporated in a current of oxygen. The mixture of vapor and oxygen was taken through a quartz tube containing platinum heated along a length of about 10 cm. to 800–900° C. and along a further length to about 400° C. From the distillate supposed to contain some NH_3 a portion was again distilled off in a high vacuum and tested, but even with this acid reliable results could not be obtained, because the acid itself would in heating yield variable amounts of nitrogen of the order of 0.5 to 2 γ (0.0005 to 0.002 mg.) N per milliliter.

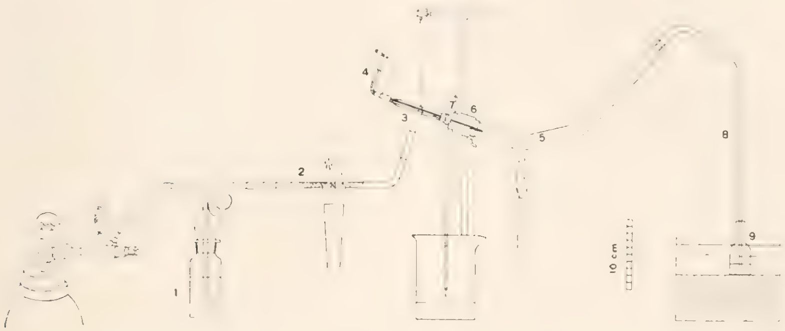

FIG. 1. Apparatus for the determination of dissolved organic nitrogen in sea water.

(1) Sulphuric acid wash bottle.
(2) Quartz reduction tube.
(3) Glass connecting head.
(4) Tube for introduction of sea water sample.
(5) Silver tube.
(6) Water jacket.
(7) Cooling water outlet.
(8) Silver condensing tube.
(9) Receiver.

The old Will-Warrentrapp method was suggested to us by Professor Biilmann and we finally succeeded in working out a modification of it suitable to our needs.

In this method the organic material is heated to about 500° C. with alkali in an atmosphere of hydrogen and, in the absence of oxygen, most of the organic nitrogen is liberated as NH_3, while a small part may be driven off as amines. No type of glass and not even quartz will stand this treatment more than a few times and we therefore do the combustion in a silver tube. The ammonia (+ amine) is taken up in N/100 HBr, combined with sodium hyprobromite, and the excess hyprobromite titrated according to the method of Teorell described in detail by Krogh in the preceding paper in this journal.

The apparatus is shown in Fig. 1. Hydrogen from a cylinder is bubbled through sulphuric acid (1) to remove any traces of NH_3 and to indicate the rate of flow which should be of the order of 10 to 20 ml. per minute.

The purification of the hydrogen before it enters the silver combustion tube may be accomplished in several ways. The simplest arrangement is to bubble it through sulphuric acid to remove any traces of NH_3 and then to pass it through a quartz tube (2) with metallic copper as a contact substance heated to a dull red heat to remove traces of oxygen. The frequent presence of arsenic and antimony traces in the copper, however, require caution in its use. A safer procedure is to bubble the gas through sulphuric acid, then through a long absorption train containing pyrogallic acid, again through sulphuric acid, and finally to pass it through the quartz tube over metallic platinum foil heated to a red heat.[2]

The connecting piece (3) is glass. The solution for combustion is added from a pipette through (4). A small part of the silver combustion tube (5) is water-jacketed and the clamp (6) grips the water jacket (7), which is a short piece of brass tubing. The current of water is regulated so that the water in the jacket will just boil when the maximum heat is applied to the combustion tube.

An analysis is performed as follows. A pellet of sodium hydroxide is placed in the combustion tube (5) which is then connected up to (3) and the system is washed out with hydrogen. The water sample—usually 5 ml.—is run in through (4). A collecting bottle with 1 ml. of N/100 HBr is placed in a basin with cold water so that the narrow tip of the silver tube will just dip into the acid. The combustion tube is heated gently at first to boil off the water and finally ignited to a very dull red heat which can be observed only when the room is dark. When sea water is analysed some ammonia will become occluded in the salts, and it is therefore necessary, after cooling, to run in an additional 2 ml. of " blank " water which should contain no nitrogen, or at all events only a known small amount, and evaporate this also. Finally the whole tube including (8) is heated (below the incineration point) by moving the flame so as to bring every trace of water into the collecting bottle (9). We have found that two determinations can be made in series without opening the combustion tube, but thereupon the salts must be washed out. To do this (3) is disconnected, a little nitrogen-free water poured in and heated to near boiling. The water jacket is emptied. The tube is turned slightly up and down in the clamp and the water poured out. This is repeated once. A fresh pellet of sodium hydroxide is put in and after connecting up, 2 ml. of N-free water is added. When this has been distilled off and the tube ignited it is ready for the next two determinations.

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[2] We are indebted to Dr. Homer P. Smith for having worked out this second procedure.

The most serious difficulty in this technique is the preparation of sufficiently pure water for the washing operations. In Copenhagen water from the municipal supply could be distilled off first from an alkaline solution of permanganate and again from sulphuric acid and permanganate. In Woods Hole and in Cambridge, Massachusetts, acceptable water was prepared by distillation from strongly alkaline permanganate and then from very dilute phosphoric acid. Both distillations were carried out in a current of washed air in all-Pyrex stills.

The distillate is tested both for free ammonia and for "organic" nitrogen by the combustion method. One sample of water gave the following results:

Test for free ammonia—Titration of 1 ml. NaBrO with

$$\left.\begin{array}{ll}\text{2.5 ml. water directly,} & 1.89_5\\ \text{5.0 ml. water directly,} & 1.89_5\\ \text{7.5 ml. water directly,} & 1.89\\ \text{2.5 ml. water} + 2.25\,\gamma\ \text{N,} & 0.66\end{array}\right\}\begin{array}{l}\text{Mean} = 1.89\ \text{ml.}\\ \text{Napthyl red}\end{array}$$

$$\text{Hence 1 ml. napthyl red} = \frac{2.25}{1.89–0.66} = 1.83\,\gamma\ \text{N.}$$

Test for "organic" N—Titration of 1 ml. NaBrO with
2.5 ml. water "burnt," 1.85, equivalent to 0.04 ml. napthyl red,
5.0 ml. water "burnt," 1.80, equivalent to 0.09 ml. napthyl red,
7.5 ml. water "burnt," 1.77, equivalent to 0.12 ml. napthyl red,
15 ml. water "burnt," 0.25 ml. napthyl red.

Hence 1 ml. of the "blank" water contains 0.030 γ N (0.030 mg. per liter). The single determinations show that within limits of error of the titration the nitrogen obtained as ammonia is proportional to the quantity of redistilled water used.

When 2 ml. of "blank" water are used in a combustion of sea water they necessitate a correction of 0.06 nitrogen or 0.03 ml. napthyl red to be subtracted from the value obtained. Distillation of deep sea water gave a satisfactory result, *i.e.,* a low and constant "blank" value.

A series of determinations on the same sea water taken in varied quantities gave the following results:

Blank value	1.90		1.88	ml. napthyl red	
2.25 γ N	0.65_5		0.67		
	1.24_5		1.21	1 ml. napthyl red = 1.83 γ N	
					Sum
Vol. of sea water, ml.	5.25	5.1	3.0	7.0	20.35
ml. napthyl red used (corrected for NH_3 in "blank" water)	0.73	0.72	0.47	1.005	2.925
γN in 10 cc.	2.54	2.58	2.86	2.62	2.64, or 0.264 mg./l.

From this quantity the amount present as preformed NH_3 must be subtracted to arrive at a figure for " organic " nitrogen.

Some control determinations on dilute solutions of glycine, creatine, and urea averaged about 5 per cent too high, but the correction for the organic matter in the distilled water used was somewhat uncertain. Analyses on acetamide and cocaine hydrochloride solutions also proved the efficiency of the method. Nitrates do not affect the results in the least. Table I, below, summarizes the results from a number of control experiments. In addition some results on various distilled waters are given.

Table I

Control analyses for organic nitrogen. Concentrations are in γ of nitrogen per liter (mg. per cubic meter).

Cocaine solutions		Nitrate solutions		Ordinary laboratory dist. H_2O	Conductivity water
Known	Found	Known	Found		
800	792	250	0	90	44
"	820	"	10	94	58
"	836	"	−6	85	40
"	798	"	14	87	46
"	778	"	4		
"	816	"	12		
400	410				
"	400				
"	388				

The Determination of Organic Carbon

We have worked out a wet combustion method not very different in principle from that described by Lieb and Krainick (1931).

The water sample is acidified to drive out preformed CO_2. Most of the chlorine is precipitated out with thallium sulphate and the water thereupon evaporated to dryness. The dry residue is oxidized with chromic and sulphuric acids in a current of carefully washed air which carries the evolved CO_2 and CO through an oxidizing combustion tube and then into a baryta solution which ultimately is titrated with HCl.

The necessary reagents are prepared as follows:

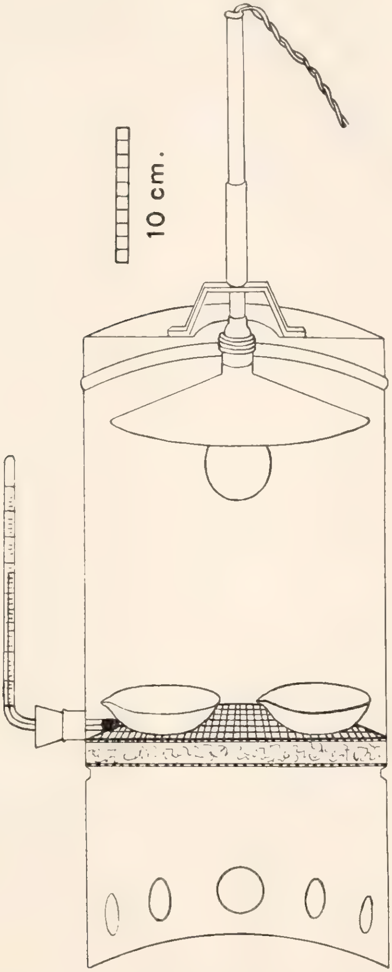

FIG. 2. Radiation evaporation chamber.

(1) Phosphoric acid dissolved in pure redistilled water and diluted to about n/10.

(2) Methyl red used as indicator.

(3) Thallium sulphate (Tl_2SO_4). The purest preparation available is melted in a crucible and stirred well so as to remove by combustion

any trace of organic material. We use for stirring a glass tube connected with an oxygen cylinder, but this may not be necessary. After cooling, the salt is carefully powdered and preserved for use in a wide-necked glass-stoppered bottle.[3]

(4) Chromate-sulphuric acid for oxidation: 10 grams potassium bichromate, 4 grams silver chromate, and 1 gram chromic acid are mixed in a crucible and melted together. The resulting solid block is pulverized.

To 50 cc. redistilled water and 50 cc. of the purest sulphuric acid we add 6 grams of this chromate mixture and 3 grams cerium sulphate. The mixture is put into the storage bottle (Fig. 3), placed in a glycerine bath at 125°–130°, and heated under aeration with CO_2-free air for about 24 hours. The mixture is kept protected against CO_2, as shown in Fig. 3 (*a*).

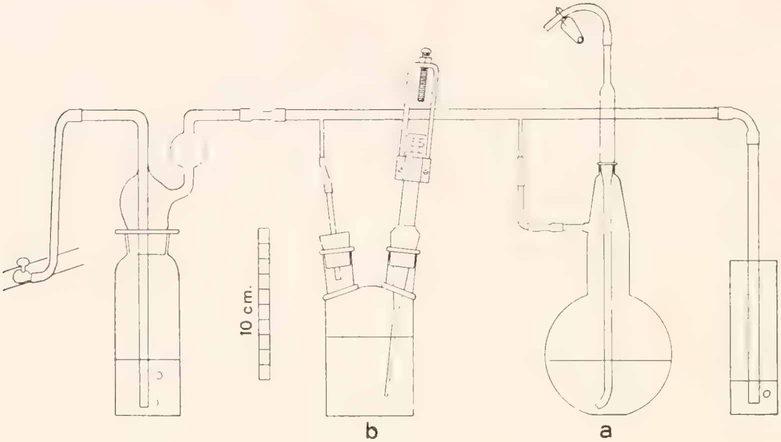

FIG. 3. Arrangement for storing reagents.
(a) Ceric-chromic sulphuric acid.
(b) Baryta solution and syringe-pipette.

(5) Potassium bichromate c.p. kept as a fine powder in a glass-stoppered bottle.

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[3] The thallium chloride precipitate from the analyses can be recovered as thallous sulphate easily. The precipitate is gathered into a large round-bottomed centrifuge tube in which it is washed and centrifuged several times, minimal amounts of cold distilled water being used for each washing. The washed precipitate is placed in a casserole with a 50 per cent excess of pure sulphuric acid and the whole cooked at 400° C. for 24 hours in a hood. The casserole should be covered with a cover propped in place so as to give a free clearance of about an eighth of an inch all around. After this digestion the temperature is raised to boil off all the sulphuric acid (450° C.) for about 6 hours. After cooling in a desiccator, the solid mass of thallous sulphate is broken out and carefully pulverized in a mortar. It is now ready for use.

(6) Hydrochloric acid N/3 used for titration in a Rehberg microburette. One cubic millimeter of this acid corresponds to 2γ carbon. The stock of acid should be kept in a bottle provided with a ground cap over the stopper to insure against evaporation, which will often take place to a quite appreciable extent from ordinary glass-stoppered bottles.

(7) Baryta solution about N/12 to N/15 prepared with 0.5 per cent $BaCl_2$ (and cresolphthalein[4]). The baryta solution is kept in a bottle protected as shown in Fig. 3 (*b*) and with a 0.5 ml. syringe-pipette mounted on top. The volume of baryta delivered by the pipette is regulated so as to correspond to between 90 and 100 mm.^{3} of the n/3 acid.

The apparatus and procedure is as follows: The salinity of the water is determined. (A hydrometer will give sufficient accuracy and all samples of ocean water can be taken as having a salinity of 35°/₀₀.) From 10 to 25 cc. of the sea water are measured off in a white porcelain basin, methyl red is added, and standard phosphoric acid is run in from a 2 cc. burette or measuring pipette until a persistent red color is obtained. The corresponding volume of H_3PO_4 N/10 is added to the samples to be analyzed. These samples (we use 25 ml. each) are measured off in centrifuge bottles as shown (Fig. 4, *c*) and thallium sulphate is added. The quantity of Tl_2SO_4 necessary depends upon the salinity. We allow an excess of about 30 per cent, which means that for a 25 ml. sample of ordinary sea water 2.5 grams of pure thallous sulphate is sufficient; a greater excess is not harmful.

The vessels are shaken in a suitable shaking machine for 10 or more hours. The shaking need not be violent. Good results have been obtained by putting in two glass balls and placing the bottles on a slowly rotating vertical wheel. After the shaking the bottles are centrifuged for a few minutes and 20 ml. of the supernatant fluid can now be pipetted off for the analysis.

These samples are evaporated down to dryness in porcelain basins. Several methods have been tried. With evaporation in an oven at 105°, splashing is difficult to control and there is some danger of losing organic matter. Evaporation in a vacuum oven is a slow process and some splashing generally occurs. The simple radiating oven shown in Fig. 2 has been found very useful. It is made from tinplate, the surface of which reflects much light and heat. A rapid current of air is maintained by the heat of the 60-watt lamp, and as the air is filtered through the layer of cotton wool, contamination with dust is effectively guarded against. The temperature may rise to 70° C., but not until the samples are almost dry, which takes about 4 hours. Eight hours will ensure thorough desiccation. If it is necessary to leave the samples for some time after drying, they should be kept well protected against moisture and dust—preferably in a desiccator.

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[4] Enough cresolphthalein is added to give a deep red color in the bottle and to give a pale but very distinct red when placed in a tube of 6-mm. bore.

The white crust of salts should be fairly easy to scrape off. This is transferred with great care to a combustion flask with side arm. We put in a couple of glass balls and one spoonful (about 0.2 gram) of potassium bichromate. The combustion flasks are cleaned with thiosulphate solution, then with bichromate cleaning fluid, and finally are rinsed just before use with a little redistilled water which is poured out as completely as possible and dried. This is done quickly by immersion in hot water after connection with a filter pump (Fig. 4, *b*).

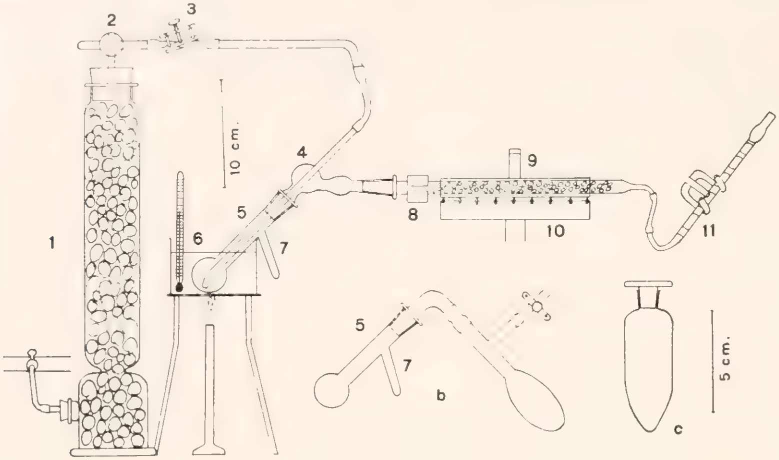

FIG. 4. Combustion apparatus for the determination of dissolved organic carbon in sea water.

(1) Soda lime tower.
(2) Three-way tap.
(3) Pinchcock air-flow regulator.
(4) Connecting head.
(5) Combustion flask.
(6) Glycerine bath.
(7) Side arm to hold oxidizing reagent.
(8) Pyrex combustion tube.
(9) Tube for thermometer in metal jacket.
(10) Long burner.
(11) Baryta tube.
(b) Arrangement for drying flasks rapidly.
(c) Stoppered centrifuge tube.

When the combustion material and bichromate have been thoroughly mixed, the suitable volume of chromate-acid is put into the side arm (7, Fig. 4). For a sample of ocean water 3 ml. are necessary. The standard ground joint is lubricated by just touching it with the tip of the pipette delivering the chromate acid.

The flask is connected with the combustion apparatus shown in Fig. 4, and the beaker with glycerine (6) used as a bath is put in position. Air at a low pressure (*e.g.* from a reversed filter pump acting as a blower) passes through moist soda lime in (1) to remove CO_2 and sweeps through the entire system.

The combustion tube (9 in Fig. 4) is arranged to remove completely all last traces of halogens or halogen acids and to ensure that any CO will be converted to CO_2. In traversing it the air passes, successively, asbestos shreds, lead chromate dispersed in asbestos, cupric oxide, asbestos, platinum foil, asbestos impregnated with finely divided metallic silver, and finally asbestos shreds. The combustion tube is made from Pyrex or other hard glass. A length of 14 cm. is surrounded by a copper tube (9) heated by the burner (10). A thermometer can be placed in (9) and the burner regulated once for all so as to give a temperature between 400 and 450° C., which is maintained throughout when analyses are in progress.

At first a fairly rapid current is maintained to wash out the system for about 10 minutes. The flow of air is regulated by the clip (3) and may be observed in the absorption tube (11), which at this stage contains a single drop of water. During this period the glycerine bath is slowly warming.

When the temperature in the glycerine bath has reached about 80–90° C., the air current is reduced so far that baryta solution can be put into the absorber (11) without danger of its being spilled out. The combustion flask is now turned so as to transfer a little of the chromate-acid in the side arm to the bulb. This procedure must be carried out with some caution because it lowers the temperature and absorbs any water vapor present and is therefore liable to suck back baryta from (11) into (8), which of course will spoil the analysis. When all the chromate-acid has been transferred it is often necessary to mix the contents in the bulb by several times revolving the tube (5). When a temperature of 120° is reached the combustion is continued for 30 minutes, which will be sufficient to drive off all the CO_2 formed.

A clip is now placed on the rubber tube of the absorber, which is transferred to a Rehberg micro-burette stand and connected with a slow current of CO_2-free air. During the titration the tip of the micro-burette dips about halfway down in the tube and the baryta is kept moving up by means of the air current. When the end point is almost reached the absorber is removed from the burette, the solution is rinsed about in the narrow portion of the absorber and afterwards in the bulb, the absorber is replaced, and the titration is finished. The end point can be judged with an accuracy of 0.1 mm.[3]

Blank determinations on the chromate-acid alone and with thallium sulphate are necessary. The chromate-acid will always give off a certain amount of CO_2 corresponding to 2–10 γ carbon, and since this liberation depends to some extent on time and temperature, it is essential that the blanks should be treated in every way like the analysis proper. The average of several blank titrations should be used for the determinations.

Example: baryta titrated directly 93.0, 93.0 mm.^{3} N/3 HCl. Blanks with 3 ml. CrA 88.1, 87.8, 89.5, 87.4, 89.3, 89.3 = 88.6 average. Samples of sea water, 25 meters, samples 1–2 untreated, 2–4 sterilized with several drops of $NaHSO_3$. The untreated samples require 0.50 ml. of H_3PO_4 N/10, the others are already acid. The same amount of Tl_2SO_4 added to each.

Titrations, mm.^{3} N/3 HCl:
Untreated 67.0, 63.1; sterilized 64.4, 63.6.
Average 64.5; blank 88.6.
Carbon found $2 \times 24.1\,\gamma$ in 20 ml. = 2.41 mg./liter.

The accuracy to be obtained does not exceed 0.1 mg./liter. When, as in Copenhagen, there is much coal dust in the air, it is essential that the combustion apparatus be kept closed except during the short intervals of time when combustion flasks are changed.

A recent series of blanks with the new mixture, containing cerium sulphate, but prepared from ordinary pure sulphuric acid, gave the following values for 0.5 ml. chromate-acid: 0.7, 1.2, 1.0, 0.7, corresponding to 3.6 γ C per ml. These low blank values indicate that the preparation of special sulphuric acid may not be necessary.

Organic Matter in Colloidal and in True Solution

The problem of the amount, if any, of organic material in sea water present in the colloidal state is of considerable importance. It is to be expected that the disintegration of organisms will yield a considerable proportion of colloidal material, but whether this will remain in the colloidal state for any length of time is at present unknown. The nitrogen determinations described above will comprise everything present, whether suspended, colloidal, or dissolved, but the precipitation of chlorides, which is a necessary initial step in the carbon determinations, will throw down all suspended particles and may be expected to remove also a variable proportion of colloids.

Colloids can be removed from water samples by ultra-filtration, but care must be exercised to guard against contamination from the filters and the filtering apparatus. We have used ultrafilters from " Membranfilter Gesellschaft," Göttingen, Germany, designated as 70 minutes, which means that at a pressure difference of 1 atm. 100 ml. of pure water will be filtered through 100 sq. cm. in 70 minutes.

Our filtering apparatus is a modification of the Thiessen Ultrafiltration apparatus and it will stand a pressure of about 5 atm. The filtering surface of 4 cm.^{2} will therefore allow the filtration of about 15 ml./hour. We generally pass the water first through a 25–35 sec. filter which will remove all suspended matter including bacteria.

If the water contains more than a trace of colloids the rate of filtration through a 70-minute filter at a constant pressure will gradually become reduced, but in the few experiments so far made with ocean water no decrease in rate was observed and no difference could be found between carbon or nitrogen values before and after ultra-filtration.

The detailed results of the application of these methods to sea water collected from various localities and depths will be presented in separate papers.

Summary

A method for the determination of organic nitrogen in solution in sea water is described in detail. The principle of Will-Warrentrap, involving digestion at 500° C. with caustic soda in an atmosphere of hydrogen, is used. The sensitivity of the method is roughly 0.0003 mg. N, giving an accuracy in sea water of about 5 per cent in 5- or 10-ml. samples.

A wet combustion method for the analytical determination of dissolved organic carbon in sea water is presented. Halides are removed with thallous sulphate, the evaporated sea water residue is oxidized by means of ceric-chromic sulphuric acid, and the evolved carbon dioxide eventually determined by acid titration of baryta solution through which the gases have passed. The accuracy approaches 0.1 mg. carbon per liter.

Differentiation between organic matter in colloidal and in true solution is made by analyses before and after ultra-filtration, using " Membranfilter Gesellschaft " collodion filters and a modification of the Thiessen ultrafiltration apparatus.

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STUDIES IN THE LIFE HISTORIES OF EUGLENIDA[1]

IV. A Comparison of the Structure and Division of Distigma Proteus Ehrenberg and Astasia Dangeardi Lemm.

A Study in Phylogeny[2]

JAMES B. LACKEY[3]

Introduction

The Euglenida are perhaps the most widely investigated of the non-pathogenic flagellates. This interest is due to their beauty, wide distribution, size, amenity to culture, and their varied modes of life, ranging from the holophytic through the saprophytic and parasitic to the holozoic. Despite our long acquaintance with them, they occupy an anomalous position in taxonomic studies, both among zoölogists and botanists, and the classification within the group is unstable, as shown by Rhodes (1926), Hall and Jahn (1929), Calkins (1933), the writer (1934), and others. In 1931, while investigating the relations between environmental factors and mitotic behavior in cells under a Grant-in-Aid from the National Research Council, the writer found an easy method of cultivating these and other Euglenida; so the present paper is an indirect contribution to that study and is a further attempt to add to our knowledge of the Euglenida.

Material and Methods

The organisms studied were obtained in activated sludge from the Tenafly, N. J., Sewage Disposal Plant, where they occur in small numbers. They were cultivated on various tap water dilutions of autoclaved wheat in Petri dishes, under a film of paraffin oil. Cover glasses with zoögleal films containing them were fixed in Schaudinn's, Gilson's, and Flemming's fluids and stained with iron hæmatoxylin, eosin being occasionally used as a counterstain.

Description of the Animals

Distigma proteus (Ehrenberg) is briefly described by Ehrenberg (1838), Walton (1915), and Pascher (1913), so no lengthy description need be given here; but in addition to the characteristics noted by these writers there are certain features which might be called to attention as additional diagnostic features. Figure 1 shows the normal type of living animal, and Fig. 2 shows the characteristic contortions which are more pronounced in this organism than in any other euglenoid with which this writer is familiar.

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[1] Journal Series paper N. J. Agricultural Experiment Station, New Brunswick, N. J., Dept. Water and Sewage Research.

[2] This is the first of a series of papers prepared through the assistance of a Grant-in-Aid from the National Research Council.

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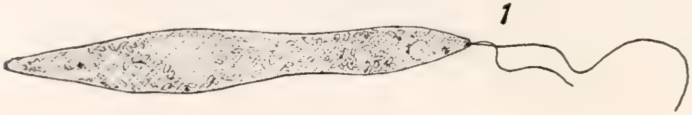
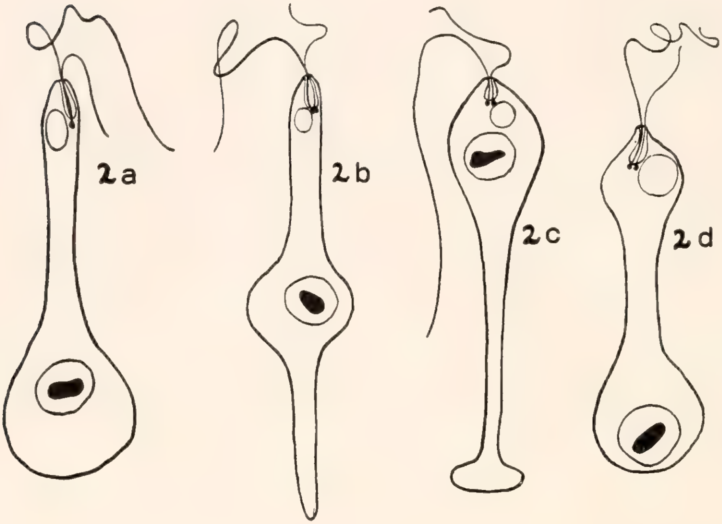




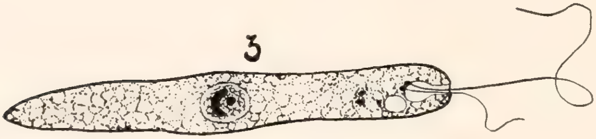


Fig. 1. Living *Distigma*, 1900 diameters; all figures are drawn at this magnification; Spencer Lens Co. apochromatic equipment having been used throughout. Note: faint gullet, reservoir, and adjacent contractile vacuole. The small size of the paramylum bodies is characteristic.

Fig. 2. Sucessive shapes assumed by a living animal as an enlargement of the posterior end of the body moves forward.

Fig. 3. Optical section of an animal after fixation in Gilson, stained with iron hæmatoxylin. Note: flagella, blepharoplasts, the endosome and accessory smaller granule in the nucleus, gullet, reservoir and contractile vacuole. Below the contractile vacuole are two cytoplasmic granules whose function is unknown.

The paramylum granules are notably constant in size and shape. Except for the one mentioned below, they seem to be the only cytoplasmic inclusions, for wherever they are absent the cytoplasm is finely granular and homogeneous.

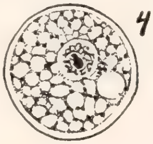
The gullet, reservoir, and contractile vacuole are shown in Fig. 3. The reservoir is quite small, and the gullet is short. One, or often two, small contractile vacuoles are formed beneath it. The two flagella enter the gullet and pass into the side wall of either the gullet or the reservoir. They do not bifurcate and each ends in a blepharoplast adjacent to the wall. Studies thus far have failed to reveal any fibrils from the blepharoplasts to any other parts of the cell. After fixations with Gilson's fluid, one or two cytoplasmic granules (Fig. 3) are found in 85 per cent or more of individuals studied, but may not be found after Schaudinn's, Flemming's, or Benda's fluids. In some they are between the nucleus and the anterior end; in others, posterior to the nucleus. It is possible that these granules are comparable to the one described by Baker (1926) as a parabasal homologue, but it has not been possible to trace them in division. Gilson's fluid also gives a different idea of nuclear structure, in that it shows an additional intranuclear body, smaller than the endosome, but present in about 98 per cent of the fixed animals. The percentages given are based on counts of one hundred individuals encountered one after the other in moving the slide across the field of vision, and are illustrative of the thousands on each cover glass. Multiple endosomes are common in the Euglenidæ, as reported by the writer (1929), Ratcliffe (1927), and others, but the selective fixation evidenced here is unique, and indicates the presence within the nucleus of two " endosomal " bodies which are of different composition. The endosome, as revealed by Schaudinn's and Flemming's fluid, is single and generally spherical or ovoid, devoid of granules or internal vesicles.

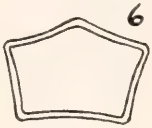
The nuclear membrane is thin, or lacking, but the nuclear vesicle is clearly defined, because the spaces in the chromatin reticulum are devoid of visible contents, apparently being filled with nuclear sap. Tschenzoff (1916) believed the nucleus in *Euglena viridis* to be a vesicle whose boundary did not constitute a definite wall. The chromatin is in the form of granules, apparently forming a permanent spireme, the granules composing linear aggregates.

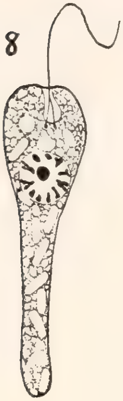
The length of the animal varies, but such great variations as mentioned by Walton and Pascher were encountered only in animals which were elongating or contracting excessively. The average length was 80 microns.

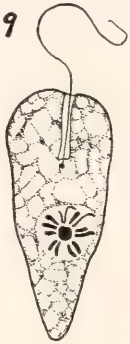
No evidence was found indicating other than a saprozoic mode of existence.

Encystment was noted in some of the cultures, but was never very common. The animals first rounded up and became quiescent while retaining their flagella. The vacuoles functioned for some time, but finally a thick wall was formed. At this time the cyst is round (Fig. 4), and the staining power is retained, but shortly after, the cyst becomes a yellow rectangular or pentagonal shape (Figs. 5 and 6) with thick walls and pores at the corners. It contains many paramylum granules, and the contents stain faintly or not at all. In cysts in which nuclear structure can be made out, there is no endosome, and the chromatin seems to be aggregated principally against the nuclear membrane. No attempt has been made to follow the history of encysted animals. As









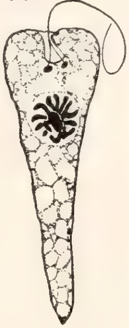


FIG. 4. Round stage of encystment. The cytoplasm is vacuolated, and numerous small black spheres are present. The wall is thick and no pores are visible.

FIG. 6. Outline, side view of a cyst similar to that shown in Fig. 5.

FIG. 8. Optical section, *Astasia*. The bifurcated flagellum ends in two blepharoplasts. The chromatin is in the form of rods, loops, or V's which do not seem to be connected.

FIG. 9. A similar section, with the flagellum seemingly not bifurcated. In reality this is a side view of it. The chromatin has a striking appearance of paired rods. It is possible to find a more reticulate appearance which is not figured.

FIG. 10. Prophase, the blepharoplasts enlarged as if dividing. The chromatin elements are exceptionally thick.

far as the writer is aware, cysts of this type have not heretofore been reported for any of the Euglenidæ.

The *Astasia* dealt with herein was obtained from the same source and cultured in the same manner and often with the *Distigma*. It appears to conform most closely to the description of *A. dangeardi*, as given by Walton and Lemmermann, and is placed in that species. The animals are extremely metabolic, whether free-swimming or creeping, and may be club-shaped one moment, a flattened oval the next. The gullet-reservoir vacuole system is so reduced as to be all but invisible in the living or stained animals at a magnification of 1900 diameters. The number of paramylum granules is small, from 8 to 12 (Fig. 7), and there is more clear cytoplasm in the animals observed than in the *Distigma*. There is a single flagellum almost as long as the body, which bifurcates in the reservoir and ends in two blepharoplasts. Hall and Jahn (1929) report that the flagellum does not bifurcate, but on slides containing thousands of individuals the writer has had no difficulty in finding plentiful evidence of bifurcation in the vegetative as well as the division stages.

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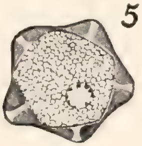

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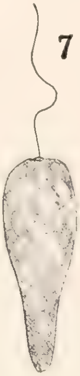

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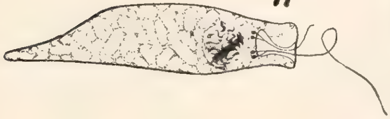

Fig. 5. Pentagonal cyst, from above. The thick yellow wall has 5 pores. The black spheres have disappeared, the cytoplasm has become homogeneous and the nucleus, devoid of an endosome, has its chromatin peripherally located.

Fig. 7. Living *Astasia*. The shape is typical and gullet, reservoir, contractile vacuole, and nuclear vesicle are faintly shown as well as paramylum granules, cytoplasmic granules, and the striations of the pellicle.

Fig. 11. Prophase, the new flagella well developed. The elongate endosome is surrounded by a thin spireme, so entangled that it is barely possible to see the endosome.

The posteriorly located nucleus seems universally to have a single endosome and the chromatin is in the form of threads (Fig. 8), loops or V's, a condition usually indicative of a permanent spireme stage.

No cytoplasmic inclusions other than the one type of paramylum bodies have been demonstrated in this study. The animals are apparently saprozoic and are facultative anaerobes. No cysts have been identified by the writer and, contrary to the statement of Walton, division is by longitudinal binary fission in the active state.

Division of Living Astasia

From cytological examination, it had been ascertained that the anterior end of a dividing *Astasia* was relatively square in shape. Watching a living culture at a magnification of 950 diameters, an individual trapped in a small space was observed at 7:20 P.M. to have this square end. This indicated approximately the metaphase. One flagellum was visible in the right half, which also showed a very small gullet and reservoir. All the paramylum was crowded into the posterior end of the body, indicating that the nucleus now occupied the anterior end. Within five minutes, a shallow median furrow appeared and deepened rapidly. At 8:00 P.M. the daughter individuals were three-fourths separated. The animal moved quite a bit within its confines, but no violent contortions occurred, as happens in *Peranema*. The new flagellum of the left half was first visible at 7:50 P.M., but thereafter, it rapidly increased in length, although it was not as apparent as the original one when the two cells parted. The paramylum granules were pushed back until the constriction was half through the body, then, nuclear division evidently being complete, the granules streamed into each daughter half. At 8:10 P.M. the two cells separated suddenly and active swimming about began.

Division in Fixed and Stained Animals

The above account is substantiated in stained preparations. The beginning of mitosis is indicated by a thickening of the chromatin threads, and the formation of the endosome into a rod. The anterior end becomes square and the nucleus migrates into it. The chromatin strands become so thick as to almost fill the nuclear space. In this organism the thickness and close spacing of the chromatin threads surrounding the endosomes is so marked as to all but obscure it and no idea of the shape of the spireme or chromosomes can be had even in very much destained specimens. They do not form an orderly pattern about the endosome (Fig. 11), as they are too long and their ends are too much entangled. Because of these conditions no definite metaphase arrangement can be made out. In the succeeding stage (Figs. 12, 13) the endosome is surrounded by a cordon of long, straight, but irregularly beaded rods, at least some of them undergoing an apparent transverse median division. Such a chromosome configuration is shown in almost all of the figures of workers who have described the division of Euglenida. Dangeard (1901) reported this, terming the rods "chromospires"; Bělař (1916) has reported a similar case of Astasia; and Khawkine (1886),

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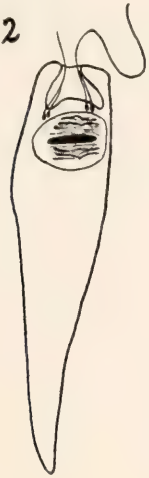

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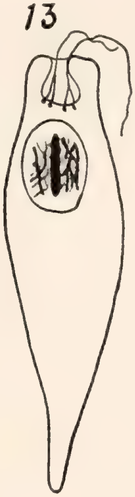

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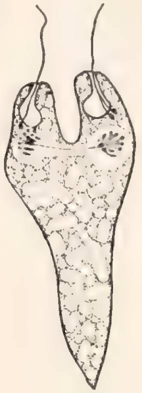

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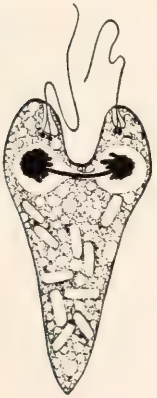

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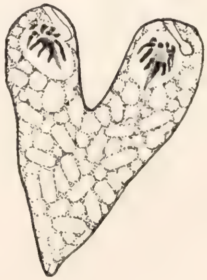

FIG. 12. A metaphase in which the chromosomes have become straight rods, which will shortly break transversely, the breaks being at the apex point of the original V-figure.

FIG. 13. Same, but the long axis of the nucleus parallel to that of the animal. Such figures are often found but no intermediate angles between this and that of Fig. 12 have been found.

FIG. 14. Anaphase. There are 12 visible daughter chromosomes at one pole, 11 at the other.

FIG. 15. Anaphase, in which the chromosomes are clumped.

FIG. 16. Telophase. Each daughter nucleus contains 12 chromosomes. No flagellla were visible in this organism.

Tschenzoff (1916), Baker (1926), and Ratcliffe (1927) have shown similar conditions for species of *Euglena;* Hall and Powell (1928) and Brown (1930) for *Peranema;* and the writer (1929) for *Entosiphon.*

The daughter groups of chromosomes move to the opposite ends of the nucleus quickly. From the observations on the living animal, it is judged that from the splitting apart of the chromosomes until the nuclear reorganization takes only about an hour. These daughter chromosomes do not become vacuolated; they simply form a tangled mass about the end of the endosome until individuality is lost (Fig. 15). It is only after the V's have become practically straight, *i.e.* when the two daughter chromosomes are still attached to each other at the end, that a correct count of their number can be made, and even then a dividing nucleus must be found in which the long axis of the endosome is vertical. Better counts may be made from anaphase or telophase nuclei. From such nuclei (Figs. 14, 16) it is believed that the number of chromosomes is 12.

The endosome, here as in other Euglenida, acts as the central axis of the division figure. In the prophases it becomes a straight rod (Figs. 11 to 16), then elongates and begins to pull apart in the middle, coming to resemble a double-headed club, which is finally pinched in two as the nuclear membrane separates. Its two pointed rods persist for some time, but eventually they shorten and enter into the reorganization of the nucleus.

The behavior of the flagellum and kinetic elements is typical. One set is retained by one daughter cell, the other getting a new set. The old blepharoplasts each bud off a new one, and the two pairs slowly separate. Evidently the roots of the new flagellum growing out from the daughter blepharoplasts unite after they have grown out a short distance. Direct evidence on this is very hard to obtain, but it is indicated in Figs. 10 and 12. The blepharoplasts in *Astasia* can hardly be considered as the division centers because of their position, which is not at the ends of the division figure, but more on the anterior face of the persistent nuclear membrane. Additional force is lent this interpretation by the retention of one flagellum and its activity, while the animal is dividing.

Nuclear reorganization is also typical. The daughter chromosomes aggregate about the end of the shortening endosome, and insensibly change into the condition characteristic of interkinesis. Throughout the whole process the cell has retained its elongate shape and is active.

DIVISION IN DISTIGMA

In this animal the cell rounds up and undergoes the euglenoid movements characteristic of *Peranema* in division. The nucleus often prepares for division before the kinetic organelles. The endosome elongates; simultaneously the chromatin forms a thin spireme (Fig. 17) of ever increasing thickness. It is not a smooth spireme, but a beaded one, the chromosomes being of varying sizes. However, the beading is much less pronounced than when the chromosomes are fully formed. The threads are either more widely spaced than those of *Astasia* at this stage.

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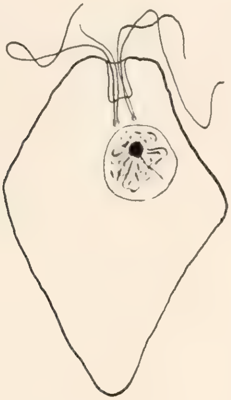

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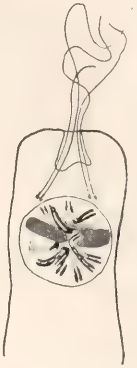

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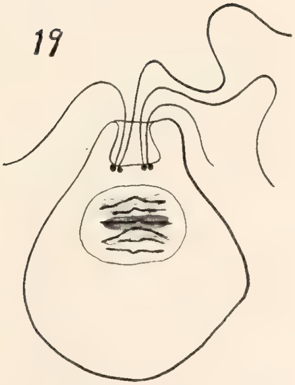

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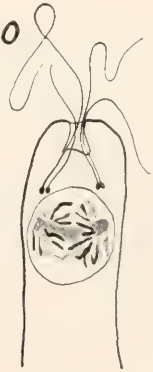

FIG. 17. Optical section transverse to the long axis of the endosome of *Distigma.* The thin spireme is well shown. Four flagella are already present.

FIG. 18. The metaphase plate in Distigma. The chromosomes are longitudinally split, and are U's or V's.

FIG. 19. Metaphase plate in Distigma. The chromosomes have straightened out to encircle the endosome as a cordon of beaded rods.

FIG. 20. Anaphase in *Distigma.* At least one of the chromosomes has not yet parted at the apex of the V. The blepharoplasts are manifestly not the poles of the division figure.

or their length and number of twists and turns is less, for whereas in a stained *Astasia* the nucleus is a mass of stained black threads, in *Distigma* the individual threads can be discerned. These threads now shorten and at the same time become thicker. Then the chromosomes are differentiated. They are oriented about the center of the endosome (Fig. 18) in a somewhat tangential plane at this time, but their length prevents the formation of a well-defined metaphase plate. From studies of this stage, it is indicated that the chromosomes are long irregular rods and that they undergo a longitudinal split. Such a split may be observed in several chromosomes in Fig. 18. When this split occurs could not be determined in *Distigma;* the spireme is too tenuous and the resulting nucleus (Fig. 17) gives no indication of paired strands of chromomeres. Tschenzoff (1916) found an anaphasic or telophasic split for the chromosomes of *Euglena viridis,* which infers an interphasic persistence of the chromosomes. Nothing of the sort can be seen here; the first appearance of a split is at the metaphase. Evidently the chromosomes split longitudinally and a separation of daughter halves begins at one end, thus forming Y's and V's which gradually open out altogether, separation of the daughter chromosomes finally occurring at the apex of the V. This would mean a terminal traction fiber attachment in cells with spindles, such as some sea urchin egg cleavages. In *Distigma* many of the chromosomes pass to the poles as straight rods just as if they had such terminal attachments and they thus present a remarkable parallel, in this anastral division, to an astral one. They do not change in length, but about the time they have formed their cordon about the endosome (Fig. 19), that body begins to elongate, and the chromosomes part in the middle, simulating a transverse division. No pairing of the chromosomes is to be found, but it would be difficult to see, because, instead of being radially disposed to the endosome, they are now parallel to it. At this time, and in the anaphase (Fig. 22) 16 chromosomes can be counted in optical sections.

As the endosome elongates, it acts like a heavy but pliable rod; if cell constriction has begun, it may bend more and more before the advancing depression until it becomes U-shaped. The chromosomes remain grouped about its two ends, undergoing few changes other than a gradual shortening. They remain separate and deeply staining after the endosome has pinched in two. Reorganization of the daughter nuclei, however, lags far behind cell division; and often daughter cells may be seen (Fig. 23) in which the pointed endosome, still appearing to pierce the nuclear membrane posteriorly, and the daughter chromosomes may still be found, marking that cell as a young individual. No especial significance seems to be attached to this, however, and the nucleus eventually assumes a typical interkinetic appearance.

The behavior of the kinetic elements is somewhat different from that in *Astasia.* The two flagella each end in a single blepharoplast, and in division each blepharoplast divides or buds off a new one. The new flagella then grow out quickly from the new blepharoplasts. The two pairs have assumed positions approximately at opposite poles of the division figure at the time of separation of the chromosomes. As in *Astasia* they never act as division centers, and logically it is difficult to

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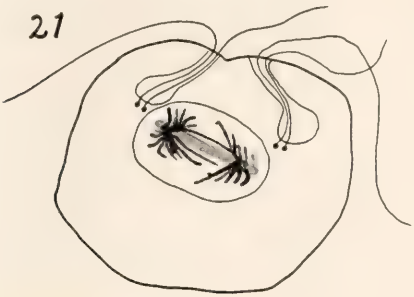

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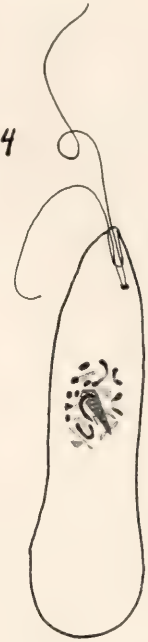

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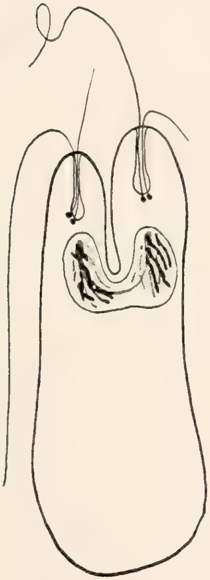

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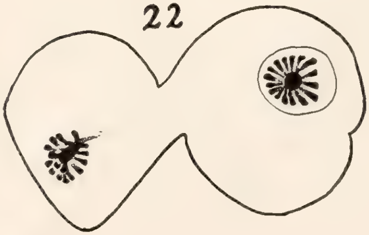

Fig. 21. Same stage, long type.

Fig. 22. Optical section through two daughter nuclei of *Distigma* at anaphase. Sixteen chromosomes can be counted.

Fig. 23. Telophase, *Distigma.* The endosome is U-shaped, and the chromosomes are still distinguishable.

Fig. 24. A "young" *Distigma.* Chromosomes still distinguishable, and the endosome still drawn to a point.

conceive how one persistent pair of blepharoplasts and another pair budded off during the process of division could act as division centers, despite the fact that blepharoplasts generally are stated to have such a function. No such assumption is necessary, however, for the progress of nuclear division and development of the kinetic apparatus are synchronous.

Discussion

The method of nuclear division in the Euglenida has been of absorbing interest since the early works of Khawkine (1886), and Keuten (1895), and the later work of Dangeard (1901) in this field. Since the Protista might reasonably be expected to offer examples of primitive modes of division, as pointed out by Wilson (1930), it is not surprising that chromospire formation in the Euglenida as described by Dangeard offered a division seemingly primitive. Recent research however, has tended to prove that the beaded chromosomes of the Euglenida in reality divide by a longitudinal split and not by a transverse break in a straight rod-like chromosome. Tschenzoff (1916) offered evidence of this, and Baker (1926). Hall (1923), Hall and Powell (1928), Ratcliffe (1927), the writer (1929), and others have shown that the chromosomes are generally of such length that they usually make up a tangled mass in the prophases and do not form an orderly, equatorial metaphase plate. However, as they split longitudinally, they begin to straighten out, so that the endosome comes to be surrounded by a cordon of elongated rods. If most of the chromosomes were rods which began to split at one end, they would eventually give a configuration such as that pictured by Dangeard in his account of chromospire formation and transverse splitting in various Euglenida, or by Bělař in *Astasia levis.* If the chromosomes were V's or if the split began in the middle, such shapes would be formed as figured by Tschenzoff, Baker, et al. In the present paper the writer believes he has shown with certainty that elongate chromosomes are formed just as in many other organisms; that a sort of metaphase plate is formed about the endosome, but the alignment of its chromosomes in an equatorial plane is obscured or prevented by their tenuousness, entanglement, and lack of spindle fibers; that each chromosome undergoes a transverse split which in most of them begins at one end and results in the formation of V-shaped structures as the split progresses; that, as the split nears the end of the chromosome, the chromosome becomes a straight rod; and that the final separation of the two daughter halves is the apparent transverse split so often seen. Bělař has shown this apparent transverse split very well in his figures of the division of *Astasia levis,* and they may be seen in the figures of other workers. If the writer's interpretation be true, however, there is nothing particularly primitive about the partition of the chromatin to the daughter cells in these Euglenida.

The position of the nucleus in dividing astasias is remarkably constant. In the interkinetic organism it may be in the anterior, median, or posterior part of the animal, but in division it is always in the anterior end in close approximation to the kinetic apparatus. If it were not for the fact that nuclear division progresses as rapidly as the division and outgrowth of kinetic elements, it would seem as if the blepharoplasts must indeed serve as division centers. A similar position is taken by the nucleus of *Euglena agilis*, according to Baker, and may be correlated in this organism with the origin of the kinetic complex from the endosome of the nucleus. However, the writer does not find any connection with, or origin from, the nucleus of the kinetic apparatus in *Astasia,* so the constancy of position is thus far without significance.

One of the most striking features in mitosis in the Euglenida is the behavior of the endosome. Spindles are present in none of the Euglenida thus far described, and it is generally stated that the endosome is the division center. In a literal sense this is true and there is little variation in the form and behavior of the endosome among the various Euglenidæ whose division has been studied. The chief variation seems to be that the endosome may change early or late in mitosis; in *Astasia,* elongation begins as soon as there is any evidence of mitotic activity; in *Distigma,* elongation occurs only when the spireme is well organized and the daughter flagella are already in evidence; and in *Euglena viridis,* according to Tschenzoff, only after the chromosomes are well formed. In forms with multiple endosomes, the elongation is occasionally shared by more than one of the fragments. Hall and Powell (1928) picture such a case in *Peranema*, and the writer has seen such instances in this same organism. The peculiarity in *Peranema* is that, when the endosome consists of more than one part, the additional fragment elongates toward one pole only, and does not divide.

In *Astasia,* the endosome quickly shortens and rounds up in the final stages of division into the shape typical of interkinesis, while in *Distigma,* the endosome retains its long-drawn-out pointed shape for some time after separation of the daughter cells and may even pierce the nuclear vesicle to enter the cytoplasm. The whole process of nuclear reorganization is very slow in *Distigma*, and one may frequently distinguish " young " cells by their chromosomes and pointed endosomes. If there is any significance in this, it is not apparent, but it does serve to call attention to the support afforded the chromosomes by the endosomes, for they retain their individuality for a long time. The writer is of the opinion that the endosome in the Euglenida takes the place of the spindle in division, and that it acts in no wise as a centriole, and does not contain a centriole in any euglenid studied by the writer.

There seems to be no definite structure which can be called a division " center " in the Euglenida. There are some accounts of centrioles, but it has not been shown that they initiate mitotic phenomena. In the Euglenida investigated by the writer, the endosome might serve as the division center in *Entosiphon,* where the blepharoplasts sometimes do not divide until metaphase, and likewise in *Astasia;* the blepharoplasts might serve this function in *Peranema;* while there is no distinguishable center in *Distigma.* Nor is the endosome a division center in *Euglena viridis,* according to Tschenzoff. In anastral mitosis, it is perhaps gratuitous to seek a division center; for there is hardly a cell structure more dynamic than chromatin itself. But it does offer difficulties to a " blepharoplast-centriole " concept, not to have blepharoplasts serving as such centers.

In view of the admittedly unsatisfactory classification of the Euglenida, the findings in this paper are offered as an aid in this field. Hall and Jahn (1929) have recently pointed out that probably the best basis for a satisfactory classification will be cytological, and they show that the Euglenidæ investigated by them all have a bifurcated flagellum, whereas the Astasiidæ do not have such a bifurcation. The writer believes, however, from a critical cytological study, that the flagellum in *Astasia dangeardi does* bifurcate. If this be true, their criterion for placing colorless forms with one flagellum in the Astasiidæ is no longer valid. However, the green members—*Euglena, Phacus, Trachelomonas,* and *Lepocinclis,* which the writer has investigated—also show the characteristic thickening on one of the flagellar roots (Fig. 25), familiar to students of the group. This structure is readily shown following fixation in Flemming's fluid and staining with iron hæmatoxylin and it is present in euglenas which have been kept either in the dark or in a rich organic medium so that they have lost their chlorophyll. If this condition is true of all the green members, and of these which have merely lost their chlorophyll, it is still possible to divide the order into families on a cytological basis. All those green members and the uniflagellate forms, whose flagellum bifurcates, and in which there is a lens-like thickening on one root of it, should be placed in the family Euglenidæ. All uniflagellate forms with or without a bifurcated root to the flagellum, but no lens-like thickening, should be placed in the family Astasiidæ, while all biflagellate and polyflagellate forms, none of which apparently show any bifurcation of the flagellum, should be included in the family Heteronemideæ. The only present change would be the necessity of transferring *Peranema* to the Heteronemideæ, close to *Heteronema*, which it greatly resembles. This scheme of classification would not invalidate the suggestions of Hall and Jahn except to make advisable the retention of the stigma-bearing astasias in the genus *Astasia* pending further cytological examination.

At present, the only colorless form with a bifurcated flagellum, aside from *Euglena*, seems to be *Astasia dangeardi*, and the writer differs from Hall and Jahn in regard to this point. There is, however, certainly no lens-like thickening on the roots or root of its flagellum, so its position is secured.

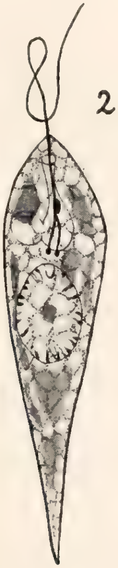

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FIG. 25. Euglena sp. A specimen fixed in Flemming and stained with iron hæmatoxylin to show the nuclear structure and the bifurcated flagellum. Each root ends in a blepharoplast adjacent to the floor of the reservoir. There is a heavy discoid thickening on the flagellum just before it bifurcates.

Moreover, such a classification represents a phylogenetic series. The ancestral form (Fig. *26a*) was probably a euglenid with chlorophyll, and a flagellum with a single root; its possession of the lens-like body is problematical, but it seems possible that the stigma is a more recently developed structure than chloroplasts. Some euglenas, notably *E. deses*, have many granules of the same material as the stigma, which move about in the cytoplasm by cyclosis or Brownian movement or both. If the stigma is a recently developed structure, so, probably, is the flagellar swelling.

From this ancestral form, the present Euglenidæ (Fig. 26*b*) arose when the flagellum split at its base, possibly as a result of abnormal behavior in division, the blepharoplast dividing, and either the flagellum attempting to divide, or the outgrowth from the new flagellum uniting with the old one. In another place the writer will show that such behavior actually occurs in *Entosiphon*.

The third step in the series resulted when *Euglena* lost its chlorophyll, and therefore the structures necessary for orientation toward a source of light. The resulting organism (Fig. 26*c*) is of the *Astasia* type. Loss of chlorophyll is so common for some species of *Euglena* that comment is unnecessary.

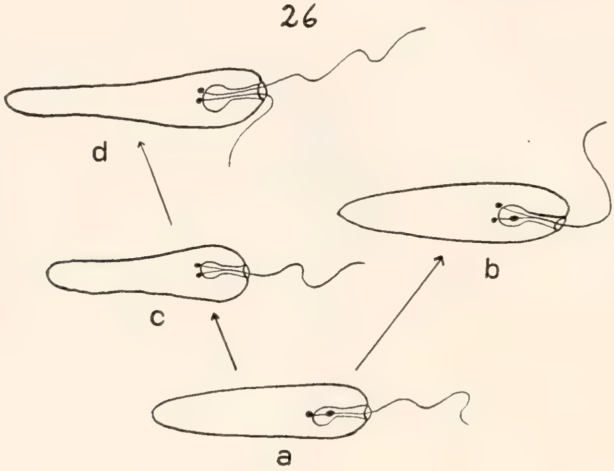

FIG. 26. The suggested phytogenetic history of the Euglenida. The primitive organism, green and with an undivided flagellum is seen at *a*. The present green forms generally conform to *b*, while the uniflagellate colorless forms such as *Astasia dangeardi* are represented by *c* and the bi- and polyflagellate colorless forms, the Heteronemideæ, represented by *d*, are derived from a form like *c*.

Finally, if an organism of the *Astasia dangeardi* type, that is, with a bifurcated flagellum root and no flagellar thickening, should have the flagellum completely bifurcated, either by a split along its entire length or by failure of the two roots to unite, the biflagellate colorless forms (Fig. 26*d*) would be produced. It is not difficult to conceive that such an evolutionary process of derivation has taken place, or even to derive such forms as *Eutreptia* and *Euglenamorpha*, and indeed Wenrich (1924) asserts that evolutionary processes are evident in his studies of the latter form, although he considers that duplication of the flagella is occurring. Such a series, however, emphasizes the interrelationships of the members of the order, and the rational basis of classifying them.

SUMMARY

1. The structures of living and stained *Distigma proteus* Ehbg. and *Astasia dangeardi* Lemm. are shown and compared.

2. Formation of yellow pentagonal cysts is noted for *Distigma*.

3. *Distigma* has 16 chromosomes, *Astasia* has 12. In both animals they are elongate and somewhat beaded. They form irregular metaphase plates and by a separation of halves of the longitudinal chromosomes, surround the endosome with a cordon of rod-like chromosomes.

4. The final parting of the two daughter chromosomes is as a rule at one end, but it simulates a transverse break.

5. Individual chromosomes may persist in the nuclei of daughter cells of *D. proteus* even after separation, but in *A. dangeardi* reorganization of the nuclei is complete by the time the cells separate.

6. Mitosis is anastral in these organisms, there being no centrioles, asters, or spindles.

7. The endosome seems to take the place of the spindle.

8. The blepharoplasts do not function as division centers, but do divide, or bud off daughter blepharoplasts.

9. One daughter cell gets an old kinetic complex (flagella, blepharoplasts) and the other part gets new kinetic organelles, the one or two flagella growing out from the blepharoplasts.

10. It is suggested that the *Euglena* with a bifurcated flagellum on which is a discoid thickening, and the Astasiidæ having a bifurcated flagellum with no discoid thickening came from an ancestral form whose flagellum did not have two roots, while the complete splitting of the bifurcated flagellum of the Astasiidæ gave rise to the Heteronemideæ, with two or more non-bifurcated flagella.

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THE EFFECT OF VERY DILUTE ETHYL ALCOHOL UPON THE RATE OF CELL DIVISION

RESULTS OF MEASUREMENTS ON EGGS OF THE MARINE WORM, URECHIS

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Most investigations of the effect of very dilute alcohol upon the rate of cell division have been carried out with *Paramecium*, but *Spirostomum, Stentor,* and *Stylonychia* have also been used. The narcotizing effect of higher concentrations of alcohol which suppress cell division has been studied on marine eggs by a number of investigators,[1] but I do not know of any attempts before now to see if there is in these forms a stimulating effect of more dilute subnarcotizing concentrations. The concentrations of alcohol which inhibit cell division in the Protozoa and in marine eggs are of the same order of magnitude, and are similar to the concentrations which cause narcosis of a large variety of animal cells and tissues: *i.e.,* about 2–5 per cent, depending on the species and the exposure time (see 3, 4, 7, 8, 9, 11, 12).

Experiments upon Protozoa

Calkins and Lieb (2) found that the division rate of *Paramecium* in $\frac{1}{25}$ per cent to $\frac{1}{16}$ per cent ethyl alcohol was increased 30 per cent or more compared with controls. The effect lasted at least a month and the general vitality of the culture was improved. Estabrook (4), however, found that concentrations of 1 per cent or less had no effect whatsoever upon the growth or division rate.[2] Woodruff (14), using pure line cultures, confirmed Calkins and Lieb in finding that $\frac{1}{25}$ per cent alcohol increased the division rate of *Paramecium,* but the stimulation was temporary (*e.g.,* 5 to 30 days) unless reëstablished by doubling the alcoholic strength to $\frac{1}{12}$ per cent, and again to $\frac{1}{6}$ per cent. Similar results were obtained with *Stylonychia.* Summarizing a large amount of data, Woodruff concludes that minute doses of alcohol decrease the rate of cell division at one stage of the life cycle and increase it (temporarily) at another.

Matheny (11) found that *Paramecia* were retarded in 3 per cent alcohol but that in concentrations ranging from $\frac{1}{100}$ per cent to 2 per cent there was no effect. Bills (1) found that dilute methyl, ethyl, propyl and butyl alcohol all improved cultures of *Paramecium* (pure lines of *P. caudatum* and *P. aurelia*) which were deficient in food. Ethyl alcohol was best. When food was deficient in the medium, in which two kinds of bacteria predominated, 1 per cent ethyl alcohol both postponed starvation of the culture and death, and revived starving cultures.

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[1] For references, see (7, 8 and 9).

[2] Retardation and inhibition occurred in 2 per cent and more.

Daniel (3) found that a certain type of *Stentor* and that *Spirostomum* thrive in 1 per cent alcohol, with the division rate stimulated. Another type of *Stentor* showed ciliary stimulation but no increase in division rate. Concentrations of 2 per cent and 3 per cent caused death in a few hours.

These results of studies on the Protozoa are in large measure inconsistent and contradictory. But this is perhaps to be expected in view of the complexity of these organisms and especially because of the variability of the media employed. It was not possible to use sterile, constant, reproducible media when these experiments were performed. Even so, there are some points of agreement throughout: (1) Alcoholic concentrations of 2 per cent or somewhat more retard cell division and growth. (2) Concentrations of 1 per cent to $\frac{1}{25}$ per cent either are without effect or else stimulate cell division and growth.[3]

Purpose of Experiments Here Reported

For testing the effect of sub-narcotizing concentrations of alcohol upon the rate of cell division, a suitable marine egg has several advantages compared with the Protozoa:

(1) The medium (sea water) is constant.

(2) There is no nutrition problem. Food is self-contained and in excess.[4] There is little reason, therefore, to suppose that alcohol will act here as a stimulant to division by compensating for food deficiency.

(3) As J. Gray (6) has pointed out, protozoan cell division is more immediately related to growth than is necessarily the early division of the egg cell, which may continue as a mere subdivision of materials, not necessarily dependent upon increase of protoplasmic mass.

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[3] Growth as well as division when the size of the daughter cells is not reduced in proportion to the increased division rate.

[4] That the rate of cell division is limited by factors other than the concentration of available food is suggested for the sea urchin egg by the results of Loeb and Wasteneys (10). That the same relations hold in *Urechis* is an assumption which is reasonable at present. The fact that eggs develop to the gastrula stage before taking in food from the outside, and (Gray (6)) that successive early cleavages proceed without diminishing cleavage rate is further evidence.

Method

The eggs of the marine worm, *Urechis caupo* (Fisher and MacGinitie (5)) have been used. One large female contains many millions of eggs which may be removed from time to time for weeks or months. All eggs used were taken from the same female, and were fertilized with sperm from the same male. The adults were kept in separate aquaria of running sea water and both yielded apparently perfect gametes two months after experiments were terminated.

All conditions were standardized as much as possible. A large automatic water bath thermostat was used which contained heating and cooling units and which varied in temperature ± 0.01° C. throughout the whole period of the experiments. Due to an error in recording, it is not absolutely certain whether the temperature of the bath was 12.93° C. ± 0.01 or 13.93° C. ± 0.01. It was almost certainly the latter, and in any case was the same throughout all measurements.

Moist chambers were made from Petri dishes in which a paraffin partition, as high as the wall of the dish, divided the bottom of each Petri vessel into two compartments. In each experiment three 1-cc. glass dishes with flat bottoms were placed in each compartment, the three on one side being the experimental vessels, the other three being the controls. Sea water covered the bottom of the compartment containing the controls, and an alcoholic solution of the concentration being used experimentally covered the bottom of the experimental compartment of the moist chamber. The cover of the Petri dish was sealed on with vaseline which also sealed the top of the paraffin partition. The moist chambers were then totally submerged and weighted down in the stirred water bath until a few minutes before the final observation time. At first a microscope was arranged to record the cleavage time while the eggs were still under water in the bath, but poor optical definition caused more error in determining the end point than the second order temperature error of removing the Petri dish and its cover and making direct observation on the stage of the microscope in the room. The room temperature was usually only 1° to 3° C. above the bath temperature, and with practice it became possible to remove the moist chamber from the thermostat only about three minutes before taking the final count. Since the measure sought was the *difference* between the time to cleavage of the eggs in the experimental and control vessels, and as these were treated exactly alike, the error due to temperature rise at this time was considerably less than 1 per cent.

All glassware and solutions were brought to the temperature of the bath before use. The solutions of alcohol were made by volume, 2 per cent, 1½ per cent, 1 per cent, ½ per cent, ¼ per cent, ⅛ per cent, 1/16 per cent, $^1/_{32}$ per cent, $^1/_{64}$ per cent, thus covering a range of 128-fold Stronger solutions than 2 per cent were of no use for the present purpose as even 2 per cent inhibited cleavage. No correction was made for osmotic pressure since alcohol penetrates the cells readily, probably to greater concentration than in the sea water medium. Eggs were, in fact, worse affected if correction was made with distilled water.

Fairly heavy insemination of a sample of eggs was carried out in a dish at the bath temperature with immediate stirring to reduce the time for sperm-egg contacts. Eggs were then pipetted into the three experimental dishes, usually between 20 and 40 eggs being dispersed in each dish to avoid crowding or proximity. This number of eggs was found to be about as many as could be accurately counted at the end. Another sample of the same inseminated eggs was transferred to a Syracuse dish containing the experimental concentrations of alcohol, but not until 5 minutes after insemination. This period in sea water was kept to permit sperm entry to be well underway before any contact with alcohol, to rule out any effect of the alcohol upon the swimming or attachment of the sperm to the egg. The first alcoholic solution served as a wash bath to avoid dilution, and the eggs were at once placed about 20–40 each in the experimental dishes containing the alcoholic solutions. The moist chamber was then sealed and placed in the tank within a minute. The eggs were out of the bath longer at the start than at the finish, due to the 5-minute delay, but the temperature error was again second order and negligible.

When the Petri dish was removed from the bath and opened it fitted onto the stage of the microscope and all six dishes in it could be observed by rotating the Petri dish. As the eggs were being observed, counts were made of the exact number in each dish, and the time was counted when that egg divided which represented division of half of the eggs in the dish. This time was estimated when there were an odd number of eggs in the dish. It was found that with a certain type of illumination an optical property of the dividing egg could be used as a very sharp end point. As the two advancing cleavage furrows approached, the thin ectoplasm lining the furrows yielded a white glow, and the time at which the glowing films of the two approaching furrows touched could be determined within 15 seconds or less in a closely observed egg. Time was usually recorded to the nearest quarter minute for a given dish of eggs. Occasionally polyspermic or unfertilized eggs were present and these were eliminated from the count, but they were very rare. In no case at any time did the controls fertilize less than 99 per cent. *Urechis* eggs are exceedingly hardy and reliable when the animals are in good condition.

Results

The results are summarized in Table I. Figure 1 is a graph of the next to last column of Table I in which the horizontal axis (alcoholic concentration) is on a geometric progression scale to magnify space between the points at the left which would be almost superimposed on a linear scale.

TABLE I

Averaged results for each concentration of alcohol. The columns, left to right, show: (1) percentage of alcohol by volume, (2) total number of experiments in that percentage, each with a control, (3) average time from insemination until 50 per cent of the control eggs had cleaved, (4) average time until 50 per cent of the experimental eggs had cleaved, (5) the percentage increase in time lapse for experimental eggs, (6) the relative cleavage rate of the experimental eggs, counting the rate of the controls as 100 per cent, and (7) the range of percentages of the individual experiments entering into the average value given in 6.

Alcohol by volume	No. of experiment	Av. time controls	Av. time experi-mental	Increase time of experiment	Experi-mental relative rate, contr. = 100%	Range
per cent		*min.*	*min.*	*per cent*	*per cent*	*per cent*
2	9	153.2	∞	∞	0	0.0–0.0
1½	18	153.2	175.9	15	87	84.2– 91.6
1	15	152.4	157.8	3.5	96.6	95.4– 98.4
1/2	18	153.1	153.9	0.5	99.5	98.0–101.0
1/4	6	152.7	152.8	0.1	99.9	98.5–101.0
1/8	9	152.4	152.4	0	100.0	99.1–100.3
1/16	6	154.2	154.9	0.5	99.5	98.9–100.1
1/32	6	151.5	151.5	0	100.0	99.8–100.3
1/64	6	150.6	151.4	0.5	99.5	98.8–100.0

It was found that the time lapse to cleavage of the control eggs, taken day after day from the same female, steadily increased.[5] On April 2, 1932, the time was 148 minutes, while by April 16 it had become 157 minutes, an increase of 6 per cent in two weeks. Partly for this reason the measurements were staggered so that those in a given concentration of alcohol were made at various times over this period of steady shift. The significant measure is the ratio of the time lapse to cleavage of the experimental and the control eggs, and this is recorded in Table I by dividing the average time for all the experimental eggs in a particular concentration of alcohol into the average time for the simultaneous controls.

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[5] Other more recent measurements using eggs from several different females recently collected suggest that the eggs from the female used in these experiments developed slightly more slowly than average. It is possible that the increased time lapse to cleavage may be due to slight infiltration of sea water into the segmental organs in which the eggs are stored once a pipette has been inserted into the gonopore as much as or more than to actual ageing or deficient nutrition of the female.

In 2 per cent alcohol complete cleavages did not take place. Beginning usually about 170 to 175 minutes after insemination, *i.e.,* well after the controls had cleaved, the eggs in 2 per cent alcohol became irregular with both large and small protrusions or lobes. The lobing was probably aided by the lowering of surface tension by the alcohol.[6] An abortive attempt at cleavage was made. The nucleus usually divided but the cytoplasm did not. At about 195 minutes the eggs rounded up and became quiescent, bearing two nuclei which sometimes fused or came to lie closely together and sometimes remained well apart. At this time the controls were in the 4-cell stage. At about 230 minutes the eggs in 2 per cent alcohol again actively attempted cell division, with lobes and protrusions, but again abortively, with only the nuclei dividing. Cleavage attempts were thus both late and unsuccessful. Eggs which remained in 2 per cent alcohol later achieved some measure of incomplete cellulation, and after 24 hours had developed cilia which slowly rotated the abnormal larvæ. Eggs transferred from 2 per cent alcohol to fresh sea water just prior to attempts at cell division largely recovered. Polar body formation was normal in 2 per cent alcohol or less, although in alcoholic solutions made isosmotic by the addition of distilled water, the polar bodies were often very large, sometimes fused, and sometimes resorbed. Isosmotic solutions were not used in the experiments, as stated earlier, because of the ready penetration of alcohol into the cells.

In 1½ per cent alcohol some protrusions and irregularities accompanied cleavage, and the blastomeres were sometimes unequal, but a very large majority of the eggs ended up completely cleaved, with smooth blastomeres and normal nuclear distribution. The criterion chosen for counting the eggs just cleaved (described in the preceding section headed " Method ") was considerably less sharply defined, and in 1½ per cent alcohol it was necessary to estimate cleavage time more roughly. Cleavage was decidedly delayed in this concentration.

In 1 per cent alcohol the cleavage type was detectably altered. The optical glow of the ectoplasm lining the furrows was less sharply defined. There was a very slight tendency for the eggs to form protrusions. The cleavage type was very close to normal, however, in all respects.

In ½ per cent to 1/64 per cent alcohol the type of cleavage could not be distinguished from that of the normal controls.

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[6] Pantin (13) has found that alcohol causes protrusions and pseudopods in a marine amœba.

Discussion

The *Urechis* egg, presumably with excess self-contained food, shows no stimulation of division rate, within less than 1 per cent, in sub-narcotizing concentrations of alcohol (Fig. 1). While it has not been directly proved that *Paramecium* and other Protozoa can use alcohol directly as a food, it is not improbable in view of its biochemical relations that alcohol can be utilized either directly or through the medium of microörganisms which may in turn be used as food. It is well established that the food supply in the medium limits the rates both of growth and cell division in Protozoa, at least when the food supply is deficient. When there is an excess of food, other factors may limit the rate of cell division. If alcohol in sub-narcotizing concentrations is utilizable as a food, directly or indirectly, the different and apparently contradictory results in the protozoan studies might be due in part at least to this effect, with different effective nutrient content of the different media. From this point of view the lack of stimulation in the *Urechis* egg is not inconsistent with the results of the protozoan studies.

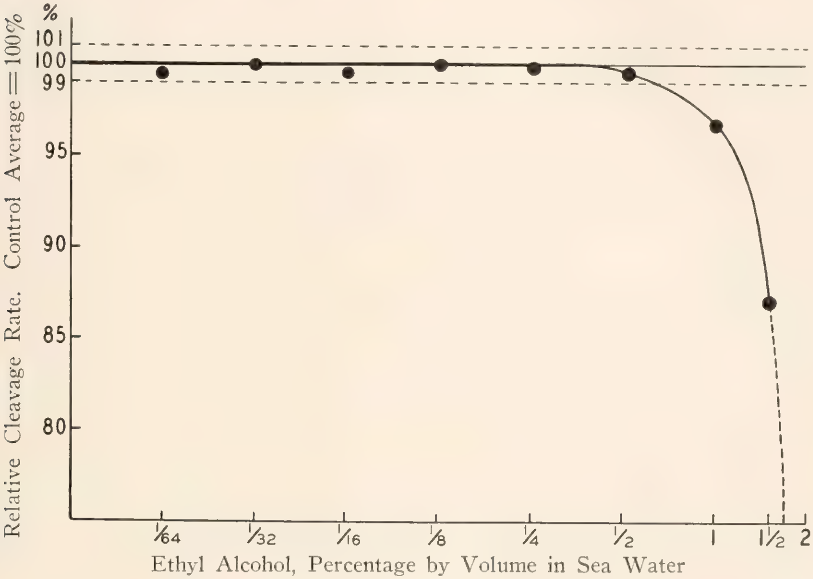

FIG. 1. The average cleavage rates (reciprocals of time lapse from insemination until 50 per cent of the eggs had just completed the first cleavage) of *Urechis* eggs in percentages of ethyl alcohol by volume in sea water. The rates are expressed as percentages of control rates. The horizontal axis is on a geometric progression scale to avoid superposition of points to the left. In 2 per cent alcohol (off scale) cytoplasmic division was inhibited.

The *Urechis* egg in constant medium with self-contained food and not necessarily dependent upon growth[7] for cell division affords a comparatively simple and direct test of the effect of dilute alcohol on the rate of cell division.

Summary

1. Alcohol has no effect on the rate of cell division of *Urechis* eggs except to retard and inhibit. In 2 per cent alcohol the first cell division is inhibited, although the nucleus usually divides.

2. In 1½ per cent alcohol the time lapse to first cleavage is increased approximately 15 per cent. In 1 per cent alcohol it is increased approximately 3.5 per cent. (See Table I.)

3. A series of sub-narcotizing concentrations of alcohol covering the range ½ per cent to $\frac{1}{64}$ per cent does not stimulate the rate of cell division nor affect it within limits of 1 per cent (see Fig. 1).

4. This covers the ranges in which stimulation to the extent of 30 per cent or more has in some cases been observed in Protozoa. In other cases, with the same and with other protozoan forms, no stimulation has been found.

5. It is suggested that the stimulation in the Protozoa, when it occurs, may be largely a nutritive phenomenon, where food supply is acting as a limiting factor to division rate. This involves the assumption that alcohol may be used as a food either directly or through the medium of microörganisms.

6. The *Urechis* egg, with self-contained food supply, presumably avoids this limiting factor. It has other points of simplicity for testing the effect of dilute alcohol upon cleavage rate, such as constant medium, which are discussed. The varying and apparently contradictory results of different investigators with the Protozoa and the results with *Urechis* eggs can be placed tentatively in agreement if certain assumptions are made.

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[7] See paragraph (3) under heading: Purpose of experiments here reported.

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FURTHER STUDIES OF EXOGASTRULATION IN THE SEA URCHIN

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INTRODUCTION

Observation has shown (Herbst, 1893; MacArthur, 1924; Runnström, 1928; Driesch, 1893) that by means of a variety of external stimuli the processes involved in the gastrulation of the sea urchin could be inhibited at the fertilized egg stage and that the potential endoderm cells subsequently formed an exovation which differentiated into a tripartite gut. In these exogastrulæ the ectoderm generally differentiated the apical plate and stomodæum while the mesenchyme frequently underwent excessive proliferation to give accessory skeletal structures and various abnormalities of the skeleton. The agents which were found to produce these effects included LiCl, $CaCl_2$, diluted or stale sea water, $CuSO_4$, HgCl, KCN, all the lithium halides, HCl, sodium butyrate, and carbon monoxide.

In a previous study of exogastrulation in the sea urchin, *Paracentrotus lividus* (Waterman, 1932), it was found that hypotonic sea water, combinations of the isotonic solutions of the chlorides of Na, K, Mg and Ca, or $MgCl_2$ alone, inhibited gastrulation in many cases, disturbed the ecto-endoblastic determination of these embryos, and stimulated excessive mesenchyme proliferation at as late a stage of development as the blastula. The types and degree of exogastrulation were comparable with those secured by other methods, especially LiCl, in which the fertilized egg was used (MacArthur, 1924; Runnström, 1928). LiCl had the same effect upon the blastula stage as it had on the earlier developmental stage except that higher concentrations of the salt had to be used (*cf.* Herbst, 1893). Driesch (1893) had previously pointed out that an elevation of temperature to 30° during gastrulation inhibited invagination.

Since these experiments demonstrated that the differential susceptibility of the blastula stage differed but little from that of the fertilized egg, it has seemed desirable to repeat the experiments with the same stages of another species (*Arbacia punctulata*) and to use different methods. To this end various stages of blastulation in *Arbacia* were exposed to hypotonic sea water, lowering and elevation of temperature, and to X-rays for varying lengths of time, after which samples were transferred to fresh sea water. Thereafter daily examination of the cultures was made.

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[1] Contribution No. 8.

EXPERIMENTAL

Hypotonic Sea Water

In previous experiments in which blastulæ of the sea urchin *Paracentrotus lividus* were exposed to dilutions up to 55 per cent, it was found that 45 per cent and 50 per cent inhibited gastrulation in some individuals and resulted in the formation of shallow exogastrulæ. The exovations never developed to any extent and in certain cases there was observable only a constriction between ectodermal and endodermal regions. Dilution of sea water up to 20 per cent but slightly retarded development while dilutions of 55 per cent resulted in death and disintegration within a few hours. As higher dilutions were used, more and more of the blastulæ failed to invaginate and the weaker ones died.

In the present experiments spherical non-swimming blastulæ gave a few shallow exogastrulæ, total inhibition of any endodermal activity, or many apical constrictions in which the apical portion of the embryo was more or less separated from the remainder. In 60 per cent hypotonic sea water the number of exogastrulæ increased, while in 65 per cent dilutions many good cases were secured. Fusions occurred at 60 per cent but did not develop. Cases of the failure of the vegetal pole cells to do anything indicate that not only was gastrulation inhibited, but likewise the division mechanism of the cells. Embryos of this type are merely hollow balls of cells with the blastocœl more or less packed with large opaque cells.

The term " shallow exogastrula " is applied to the condition in which some endodermal outgrowth occurs but the exovate is frequently irregular, never of any considerable amount, and does not differentiate the three divisions characteristic of the gut. Subsequent development is limited to apical plate and stomodæum. No skeleton forms and these abnormalities do not long survive.

Early swimming blastulæ gave a few good cases of exogastrulation at 30 per cent dilution. The numbers increased up to 50 per cent and then fell off as higher dilutions of sea water were employed. Apical constrictions were common. As the more resistant individuals were affected by the higher dilutions, the weaker ones were killed and disintegrated.

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Swimming oval blastulæ gave a few very simple exovates at dilutions of 55 to 60 per cent. Embryos which remained in 55 per cent hypotonic sea water developed both exovates and apical constrictions, while in 60 per cent excellent fusions, apical constrictions, and a few exovates appeared.

At a much later stage which shows the flattening of the vegetal pole, 65 per cent hypotonic sea water gave a very few cases of exogastrulation together with apical constrictions. Dilutions of 55 to 60 per cent gave a few typical constrictions only. Other abnormalities involved skeleton, gut, or arm formation.

An initial stage of gastrulation was also used to determine if the gastrulation process, once it was started, could be inhibited and this was followed by the external development of the endoderm. In no experiments with this stage were exogastrulæ found. Forty-five per cent dilutions gave no development beyond the triangular gastrula stage. Fifty per cent inhibited further gastrulations; 55 per cent resulted in no further development outside of a few apical constrictions; while 60 per cent caused death. At a more advanced stage of gastrulation 40 to 50 per cent dilutions gave stunted and abnormal plutei and 60 per cent gave apical constrictions but no development of arms. Retardation was in proportion to the dilutions employed.

Elevation of Temperature

Spherical, non-swimming blastulæ, exposed to a temperature of 28°–29° C. for 5 hours, continued their development during this period and when returned to normal room temperature (22.5° C.) were at the stage of flattening of the vegetal pole or initial gastrulation. No difference could be seen between them and the controls in regard to the degree of development. Twenty-four hours later most of the individuals of the culture had developed to the young pluteus stage, a relatively few had gastrulated but had remained in the globular form; in some gastrulation was abnormal, while about one per cent were definite but rather shallow exogastrulæ.

The same stage exposed to a temperature of 31–32° C. for 5 hours developed very slowly during this period. At the end of the 5 hours they were at the oval stage of blastulation and were swimming very slowly. Twenty-four hours later a few had died, most were at the triangular gastrula stage, and a few had gastrulated in an abnormal fashion. In addition there were present in the culture samples a few shallow exogastrulæ together with individuals in which the vegetal pole had ruptured giving a small mass of cells attached in this region. Forty-eight hours later the triangular gastrulæ had developed to the pluteus stage while the abnormalities had all died.

Older stages up to gastrulation were exposed to the same elevations of temperature. At 28–29° C. for 10½ hours the spherical swimming stage gave a few shallow exogastrulæ. At 31–32° C. young plutei developed after 24 hours at room temperature together with a few exogastrulæ. Many individuals had died. At 32–33° C. most of the individuals died after 24 hours in normal sea water. Gastrulation was entirely inhibited and only a few large opaque blastulæ with large swollen cells remained alive. At 33–34° C. all died.

The stage of initial gastrulation (flattening of the vegetal pole) kept at 30° C. for 1¼ hours gave mostly mature plutei with a few definite undifferentiated gastrulæ. At 32–33° C. for 1½ hours movement is slowed and 24 hours later many dead individuals were found in the cultures. The embryos either were unaffected or killed as no exogastrulæ were seen. A temperature of 35° C. for one hour killed all the embryos.

The stage of initial and earliest gastrulation was for the most part unaffected by a temperature of 30° C. for one hour. An occasional undifferentiated exogastrula occurred. At temperatures below 27° C. development appears to be unaffected.

For comparison eggs in the 2 to 4-cell stages were kept at a temperature of 30° C. for one hour. Most of these gave typical plutei in 24 hours and also an occasional shallow, irregular exogastrula. Eggs 30 minutes after fertilization exposed to 30° C. for 30 minutes developed many shallow exogastrulæ. At least 50 per cent of the embryos were abnormal in one way or another. The other stages studied were unaffected by an exposure of this strength and duration.

Lowering of Temperature

In another experiment samples from the same batch of eggs were transferred to the cold room at 2° C. at different stages of development and were kept at this temperature for varying lengths of time. Samples taken at 15, 30, and 45 minutes after fertilization, and of 2-cell, 4-cell, 8 to 16-cell, 32-cell plus, 64-cell and oval swimming blastula, were exposed to this temperature for 9 to 19 hours. Twenty-four to 48 hours after transfer to fresh sea water, stunted or abnormal plutei, globular embryos with gut and skeleton, gastrulæ, irregular gastrulæ, and blastulæ were found. A few shallow exogastrulæ occurred in the cultures of eggs which had been placed in the cold room 15 minutes after fertilization and at the 64-plus cell stage. Gastrulation was increasingly abnormal at the longer exposures, but in all cases development continued during the experiment. In some the irregular swimming blastulæ showed rupture of the vegetal pole. Oval swimming blastulæ were least affected, the younger stages more.

Swimming oval blastulæ were also exposed to 2°, 4°, and 7° C. for 24 hours. Temperatures of 2° and 4° C. gave an occasional simple exogastrula, but the majority were small plutei with a few blastulæ and abnormal gastrulæ. Young non-swimming blastulæ were exposed to temperatures of 2°, 4°, and 7° C. Five hours at 2° C. gave mature plutei together with a few globular individuals in which the gastrulation process was entirely inhibited. Gastrulation occurred during the experiment.

Similar stages kept at a temperature of 4° C. for 18 hours gave small plutei and an occasional shallow exogastrula or abnormal gastrulation. During sojourn at 7° C. for 18 hours development progressed as far as the oval, swimming blastula stage. At this time the temperature was slowly raised to 31–32° C. and maintained for 9 hours. Twenty-four hours later most of the embryos were dead without having gastrulated. A few irregular gastrulæ showed lethargic movement. Each one of these two environmental changes alone is not sufficient to affect the process of gastrulation to any significant extent, but when combined effectively prevent it.

From this data it seems evident that elevation or lowering of temperature has little influence upon the factor or factors responsible for gastrulation except, in the very weakest individuals, to make the process somewhat irregular. An occasional exogastrula may develop around 2–4° C., or gastrulation may be entirely inhibited.

X-Ray

A few preliminary experiments were also made in which both fertilized eggs (25 minutes after fertilization) and blastulæ (spherical and oval swimming embryos) were exposed to 720 revolutions per minute at 20 cm. distance for the same lengths of time, *i.e.,* 2 to 12 minutes. Samples were removed at 2-minute intervals.

In agreement with the results described above, it was found that both eggs and blastulæ vary markedly in their susceptibility, but there is little correlation between length of exposure and the amount or degree of effect as regards gastrulation. In both cases apparently normal although somewhat smaller plutei were found even after 12 minutes' exposure to the stimulus. With increasing length of exposure, more of the weaker individuals were killed and fewer others reached the pluteus stage.

Fertilized eggs exposed for 2 minutes develop blastulæ to young plutei in 48 hours. These die much earlier than the controls, indicating that their resistance has been weakened by the treatment, as normal plutei will live for days before dying of starvation. The number of plutei appearing in the cultures steadily decreases following progressively longer exposures and more individuals fail to gastrulate or never get beyond the gastrula stage. In those cases where the gastrulation process has been inhibited, the endoderm shows very little growth. Some outpushing of the endodermal cells does occur but it is very slight.

Thus not only has there occurred an inhibition of the gastrulation process but also an interference with the division rate of the cells. In other cases where gastrulation has occurred the endoderm has been affected in that it is present as large or small irregular masses of cells near the vegetal pole. The gut is very abnormal. An occasional exogastrula occurs in cultures exposed from 6 to 12 minutes but the exovation is very shallow and shows no differentiation. Other abnormalities noticed include apical constrictions and rupture of the vegetal pole similar to those described above, and development of a few giant transparent circular blastulæ lacking most of the mesoderm.

As is the case with the fertilized eggs, the effect upon the blastulæ is to retard and inhibit further development in the weaker individuals. In cultures exposed from 2 to 12 minutes more and more of the weaker individuals are affected depending upon their resistance. Effects vary all the way from death, inhibition or retardation of gastrulation, to modification of the apical portion of the body skeleton. The body rods frequently are sharply pointed. The blastula stage seems to be as susceptible as the fertilized egg stage.

A further abnormality concerned those individuals which had failed to escape entirely from the vitelline membrane. Such individuals are constricted by the partially enclosing membrane into double formations comparable to exovations, but they generally fail to gastrulate. The possible effect of the X-rays is to harden the membrane to such an extent that the enzyme produced by the embryo is not capable of destroying it, and the embryo is unable to escape entirely from the rigid shell. In cases where escape has been successful the empty membranes may persist for some time.

Embryos at the stage of the flattening of the vegetal pole (initial gastrulation) are much more resistant to X-rays than the stages just described. Cultures of this stage exposed to similar intensities and for the same length of time show fewer deaths, a higher percentage of normal plutei, and almost 100 per cent gastrulation. Only occasionally was gastrulation completely inhibited and this inhibition also involved any endodermal proliferation.

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Discussion

The methods used in the present experiments serve not only to increase the list of those stimuli which have been shown to cause inhibition of the gastrulation processes and the external development of endoderm, but also indicate that stimuli other than chemical ones may produce a similar effect although not to the same degree. Fewer cases of exogastrulation were secured in these experiments than in those previously reported for *Paracentrotus lividus,* and, moreover, very few of them underwent any further differentiation. This may indicate that either the gastrulation processes in *Arbacia* are less susceptible to environmental stimuli than in *Paracentrotus,* or the stimuli employed affected other developmental processes more adversely.

In general, the results of these and previous experiments point to a high degree of susceptibility of the gastrulation processes to changes in the external environment throughout early development. If a comparison is made of the types of exogastrulæ and their subsequent differentiation between those secured following exposure at the fertilized egg stage and those at the blastula stage in *Paracentrotus* alone, it is seen that more typical differentiation of larval organs follows the exposure of the earlier stages. In the older stages subsequent development is more abnormal and retarded. Thus, while more cases appear with the earliest stage and they show more complete subordinate differentiation, with the older stages fewer cases are secured and these are more atypical as to ectodermal and skeletal differentiations. The gastrulation processes become increasingly more difficult to disturb, while on the other hand histogenic processes are more easily affected.

Various ideas have been advanced for the explanation of exogastrulation. Some have referred to a differential growth process (Herbst, 1893; Driesch, 1893), while others have been mechanical in nature, as for instance: (*a*) differences in rate of growth between ectodermal and endodermal portions of blastula whereby a lateral pressure is exerted on the ectodermal plate; (*b*) resistance of the closely pressed egg membrane, so that the surface increase of the blastula is possible only internally; (*c*) constantly decreasing amount of fluid in the blastocœl perhaps exerts a sucking effect on the endodermal plate; and (*d*) the decreased ectodermal area (Lithium effect) is unable to accommodate the enlarged archenteron in its interior (Huxley and DeBeer, 1934, p. 334). From the available data and also from the fact that fertilized eggs, cleavage stages, and blastulæ will give exogastrulæ, it would appear that any mechanical explanation of this phenomenon is inadequate. All of these are not free of criticism since the membrane is separated from the blastula by the perivitelline space filled with fluid and the fluid within the blastocœl would have to be displaced, and further since the membrane disappears from the blastula stage of both *Paracentrotus* and *Arbacia* early in blastulation.

Recently Moore (1930) has found that an excess of Ca added to sea water causes displacement of the nuclei of *Strongylocentrotus purpuratus* blastulæ to the inside, while Li-larvæ of *Dendraster* and *Strongylocentrotus* showed nuclei in the periphery. From this he reasons that nuclear position does not correspond to invagination or evagination. In cell plates from fragmented larvæ of *Dendraster* the cell chains are bent or curved; accordingly he suggests that invagination is caused by asymmetrically placed cell bridges. If this is true, then the placement of these cell bridges can be affected not only at the fertilized egg stage before any cleavage has occurred, but also during any stage of cleavage or blastulation up to initial gastrulation when the cell bridges are already established.

Runnström does not explain the failure of the endodermal cells to invaginate while MacArthur appeals to such mechanical factors as pressure within the cavity of the blastocœl and lack of space due to the large amount of mesoderm produced by the inhibiting action of LiCl. On the whole, the cause of the invagination of the cells of the vegetal pole of the blastula is but very imperfectly understood. Consideration of the great variety of stimuli which may inhibit gastrulation and the fact that this inhibition may occur at different developmental stages seems to indicate that it is a question of an action upon a specific potentiality localized at the vegetal pole of the egg and blastula stages.

During cleavage this potentiality is segregated in the macromeres at the vegetal pole, endowing these cells with the capacity to push into the cavity of the blastocœl. Thus any agent which would act to inhibit this property in the fertilized egg would likewise affect the cells containing it at the blastula stage.

This idea of a specificity of the vegetal region is not a new one, as Hörstadius (1927) has been able to demonstrate by means of vital coloration and microdissection that one of the determining factors in the type of segmentation in *Paracentrotus* is the existence of a particular kind of autoplasm in the vegetative hemisphere of the egg (micromeres and macromeres) which acts in the capacity of an organizer upon the presumptive ectoderm. This author has also shown by a study of the developmental potencies of pieces smaller than halves, that there exists in the early embryo a distribution of potencies along the primary egg axis. At the 64-cell stage the lower group of macromeres represents potential endoderm while the micromeres contain the presumptive primary mesenchyme.

The numerous stimuli which have been found to inhibit gastrulation at the blastula stage of development act upon certain potencies of the presumptive endodermal cells in such a manner as to prevent invagination. Accordingly, the endoderm differentiates as an exovate. The gastrulation process is very sensitive to environmental changes at any stage of early development but becomes increasingly more difficult to disturb in progressively older stages. Furthermore, its susceptibility varies in different individuals of the same culture and also with the kind of stimulus. The micromeres are also affected since the mesenchyme generally undergoes precocious proliferation.

SUMMARY

1. The phenomenon of exogastrulation characterized by inhibition of the gastrulation processes, external differentiation of endoderm, and the excessive proliferation of mesenchyme, has been produced in *Arbacia punctulata* by exposure of blastula stages to hypotonic sea water, lowering and elevation of temperature, and X-rays.

2. Dilutions of sea water of 45 to 65 per cent give the most numerous cases of the various methods employed. A few occur at 30 to 40 per cent dilutions.

3. Development is typical at 27° C. but at higher temperatures becomes increasingly irregular and abnormal. A temperature of 35° C. kills the cultures or if exposure is for a short time only, inhibits any further differentiation. Shallow exogastrulæ appear at 28° to 31° C.

4. Lowering of temperature has little effect upon gastrulation. Development is fairly typical but retarded at 7° C. At 2° to 4° C. an occasional exogastrula occurs while the stronger individuals eventually gastrulate at this temperature.

5. In the X-ray experiments, dosages of 720 revolutions per minute at 20 cm. distance for 6 to 12 minutes give a few exogastrulæ.

6. Results indicate that stimuli other than chemical ones will inhibit gastrulation at the blastula stage of *Arbacia*. Great injurious effect on subsequent development is apparent since the exogastrulæ fail to differentiate except for apical plate and stomodæum. The exovation is usually shallow.

7. Fusions, ectodermal constrictions, and permanent blastulæ are common even where no exogastrulæ appear.

8. With increase in length of exposure and in strength of stimulus, the more resistant individuals are affected while the weaker ones are killed or fail to show any further development. Various stages of blastulation appear equally sensitive.

9. Examination of the long list of stimuli which will cause exogastrulation would seem to show that any mechanical explanation is unsatisfactory since this phenomenon can be provoked by exposing fertilized eggs, cleavage and blastula stages. It is concluded that some potentiality present in the vegetal pole of the fertilized egg and later segregated in the vegetal cells (presumptive endoderm) of the blastula causes the inturning of cells during gastrulation. This is very sensitive to environmental changes at any stage of development up to the time of gastrulation but varies in susceptibility in different individuals. Accompanying phenomena of cell division and differentiation are also affected according to the stimulus employed and length of exposure.

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THE LIFE HISTORY OF THE COPEPOD TORTANUS DISCAUDATUS (THOMPSON AND SCOTT)

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INTRODUCTION

During the examination of a series of plankton samples taken at Friday Harbor, Washington, over a period of about four years (1924, 1926–1929), an undescribed microscopic egg was encountered in rather small numbers but with such regularity throughout the warmer months that a record was kept of its occurrence. This record and a brief description of the egg were included in the report on the plankton investigation (Johnson, 1932). Shortly before the investigation was terminated, a number of these eggs were taken from the plankton and hatched in culture dishes in the laboratory at Friday Harbor. The hatched larva proved to be an undescribed copepod nauplius. However, it did not survive beyond the first stage and my removal to the East Coast prevented further experimentation at that time.

The work was again resumed in early September, 1933, and as a result of the study the egg was determined to be that of the planktonic copepod *Tortanus discaudatus* (Thompson and Scott). Due to the increased recognition of the importance of studying the developmental stages of animals occurring in the plankton, the successive nauplius and copepodid stages were followed through and are here described.

Tortanus discaudatus is a common neritic copepod with a wide distribution in boreal waters of the Pacific and Atlantic coasts of North America. Seldom does it occur in very large numbers, but it may assume an important rôle in neritic communities. Willey (1920) reports that it composes 50–75 per cent of the summer copepod plankton off Souris, Prince Edward Island, and in Passamaquoddy Bay at St. Andrews, New Brunswick, it was found at times to constitute over 40 per cent of the total animal community of the plankton in the warm months (Fish and Johnson, unpublished data). It is the only species of this genus recorded from Puget Sound waters.

The author wishes to thank Dr. T. G. Thompson, Director of the University of Washington Oceanographic Laboratories, for laboratory facilities and Dr. C. B. Wilson for final criticism and suggestions.

METHOD

Eggs were removed from fresh plankton hauls and placed in filtered sea water for the culture of the first nauplius stage. This stage was also hatched from eggs which had been deposited in culture dishes by adults taken from the plankton. The eggs could not be obtained in large numbers and were therefore usually kept in watch glasses that could be used directly under the microscope. Adults and older larvæ were cultured in finger bowls or section jars. An even temperature was maintained by surrounding the dishes with cold running water. All later larval stages used in the study were obtained directly from the plankton. Mixed diatoms were placed in the culture dishes but there was no evidence of the larvæ having fed upon them. If cultures of tintinnids had been available for feeding, better results in rearing might have followed, for there is evidence that these ciliates constitute a large item in the diet of the larvæ. Adults were fed copepod larvæ taken from the plankton.

All drawings were made with the aid of a camera lucida.

PROCEDURE AND RESULTS

The pelagic eggs removed from the plankton and cultured in the laboratory never failed to hatch within three days. Repeated attempts gave the same result, of which the following is typical:

September 11. Ten eggs placed in culture dish.
" 12. One egg hatched.
" 13. Five additional eggs hatched.
" 14. Four additional eggs hatched.

All the larvæ hatched from these eggs lived less than four days, none surviving to pass into the second nauplius stage. The five following nauplius stages could, however, readily be recognized and selected from the general plankton material for there is no other copepod nauplius with which it might easily be confused in this region.

The metamorphosis occurring at the critical moult between the sixth nauplius and first copepodid stages results in such drastic changes in the structure that it is not possible to bridge this gap by observations on added spines, segmentation, etc., as is practical in the individual successive stages of either the nauplii or copepodids. Oberg (1906) overcame this difficulty by removing larvæ from the plankton and rearing them experimentally through the critical moult. In the present species the general shape of the body, the color of the second antennæ, and the structure of the second maxillæ of the last nauplius stage were valuable hints that suggested a relationship to the first copepodid stage of *Tortanus discaudatus* which was recognized in the plankton tows. These hints, together with the fact that the eggs and larvæ of this species were as yet unknown, led to an attempt to obtain eggs definitely known to have been spawned by *Tortanus discaudatus.*

On October 18 ten female and eight male adults were taken from the plankton and placed in culture dishes in the laboratory. At least two of the females were obviously ripe and had spermatophores attached when they were placed in the dish. On the following day it was found that 73 eggs had been deposited in several groups of double rows as shown in Plate I, Fig. 1. The greatest number in a group was 20. Later development of the egg case showed it to be like that of the pelagic egg taken from the plankton. Only a few of the eggs made much progress in development. However, four embryos did develop, and two of these hatched to normal nauplius larvæ identical with the first nauplius resulting from the pelagic egg of the plankton. The remaining two, although active and apparently normal, were unable to escape from the case enclosing them. The natural opening of the case was doubtless prevented by imperfections in its formation. This will be more fully discussed later.

The Egg

(Plate I, Figs. 3 and 4)

The egg proper is 0.11 mm. in diameter and is contained in a case or capsule especially constructed for flotation by the development of a thin, flat disc surrounding the equator of the single egg. This disc is 0.08 mm. in width, making the whole case 0.27 mm. in width as seen from above. The egg is granular and opaque due to the large amount of deuteroplasm, and the case is semi-transparent with very fine reticulations on the disc. One side of the case is provided with a fissure which serves as an operculum to open for the escape of the larva (Plate I, Figs. 3 and 4, *o*).

When the eggs are first deposited they are spherical and the specialized flotation case so characteristic of the older eggs found in the plankton is wanting. This disc-like case is formed some time after the eggs have been cast into the water. Its early formation is shown in Plate I, Figs. 1 and 2. The first indication of its development is seen in the appearance of a number of small blister-like structures covering the egg's surface. Finally one side (apparently predetermined, as suggested by the orientation of the eggs shown in Plate I, Fig. 1) is drawn out, forming a thin plate of double thickness. This plate spreads laterally until it results in the disc surrounding the egg at the equator.

Most of the eggs deposited in the culture dish showed some progress in the development of the case but none were so completely and symmetrically formed as were the cases enclosing eggs taken directly from the plankton. One feasible explanation for the failure to form perfect cases in the cultured material is found in the fact that the freshly spawned eggs are slightly mucilaginous and therefore adhere lightly to the bottom of the culture dish. This adherence would interfere with the proper expansion of the cases. Interference of this kind would be absent in the natural planktonic environment where the spawning is directly into deep water and where the membranes have an opportunity to shape and harden while the egg is still in suspension.

The Nauplius Larvæ

In the living condition the nauplius larvæ are readily recognized by the conspicuous orange-red pigment in the outer part of the relatively long second antennæ. This coloration is present in all the nauplius stages but has a tendency to become somewhat darker as the older stages are reached. The pigmentation is also present in the mandibles though less conspicuous. The eye is dark red. Other characteristics common to the nauplius stages are: (1) the presence of a large broad labrum which is narrowed at the anterior end and indented on the posterior margin which, following the first stage, is fringed with long weak bristles, (2) the colorless, long and slender anterior antennæ, (3) the hind part of the body being straight rather than flexed ventrally as in the nauplius of some species, and (4) the anterior end of the body being angular in outline in all but the first stage.

Stage I (Plate I, Fig. 5)

Body 0.12–0.14 mm. long, dorsal aspect egg-shaped with broader anterior end; posterior end with two medium strong, slightly curved spines of equal length.

First antenna. Three segments, the first fused with the second and bearing one short ventral bristle, the second with one long ventral bristle, and the third or distal with three long terminal bristles.

Second antenna. Coxopodite with one masticatory hook. Basipodite with one very small masticatory hook. Endopodite of one segment with one lateral and two terminal bristles. Exopodite of six segments: the first completely fused with the second and with no bristle, 2–5 with one long bristle each, and the sixth with one long and one shorter bristle.

Mandible. Coxopodite with an inconspicuous blade-like process bearing a very small spine. Basipodite with two small spines on the inner side. Endopodite of two fused segments discernible only by the armature; the first segment with two short bristles and the second with three longer bristles. Exopodite of four segments, 1–3 with one bristle each, the fourth with two bristles.

Stage II (Plate I, Fig. 6)

Body 0.185–0.205 mm. long, slightly angular anteriorly, posterior end armed with one long plumose bristle and one shorter weaker plumose bristle situated at its left; the shorter (left) bristle curved ventrally. Laterad to each of these bristles is a series of very minute bristles.

First antenna. As in I, but the second segment with one long and one short ventral bristle, the distal segment with three long bristles and one shorter weaker accessory bristle at the tip.

Second antenna. Coxopodite with one very strong masticatory hook. Basipodite with one strong masticatory hook nearly as long as that on the coxopodite but more slender, and one weak short bristle on the posterior margin near the outer end. Endopodite of one segment with three long terminal and two shorter lateral bristles. Exopodite of six segments, the first fused with the second and bearing no bristle, the second with one long and one short bristle, 3–5 with one long bristle each, the sixth with one long and one short bristle.

Mandible. Coxopodite as in I, but with spine more pronounced. Basipodite with three spines on inner side. Endopodite segments as in I, the first with a strong hook and two bristles directed inwardly, the second with four posteriorly directed bristles. Exopodite as in I, but with one long and one short bristle on the first segment.

Stage III (Plate I, Fig. 7)

Body 0.23–0.26 mm. long, the posterior end bearing two long terminal spines and between them two longer plumose bristles (the left

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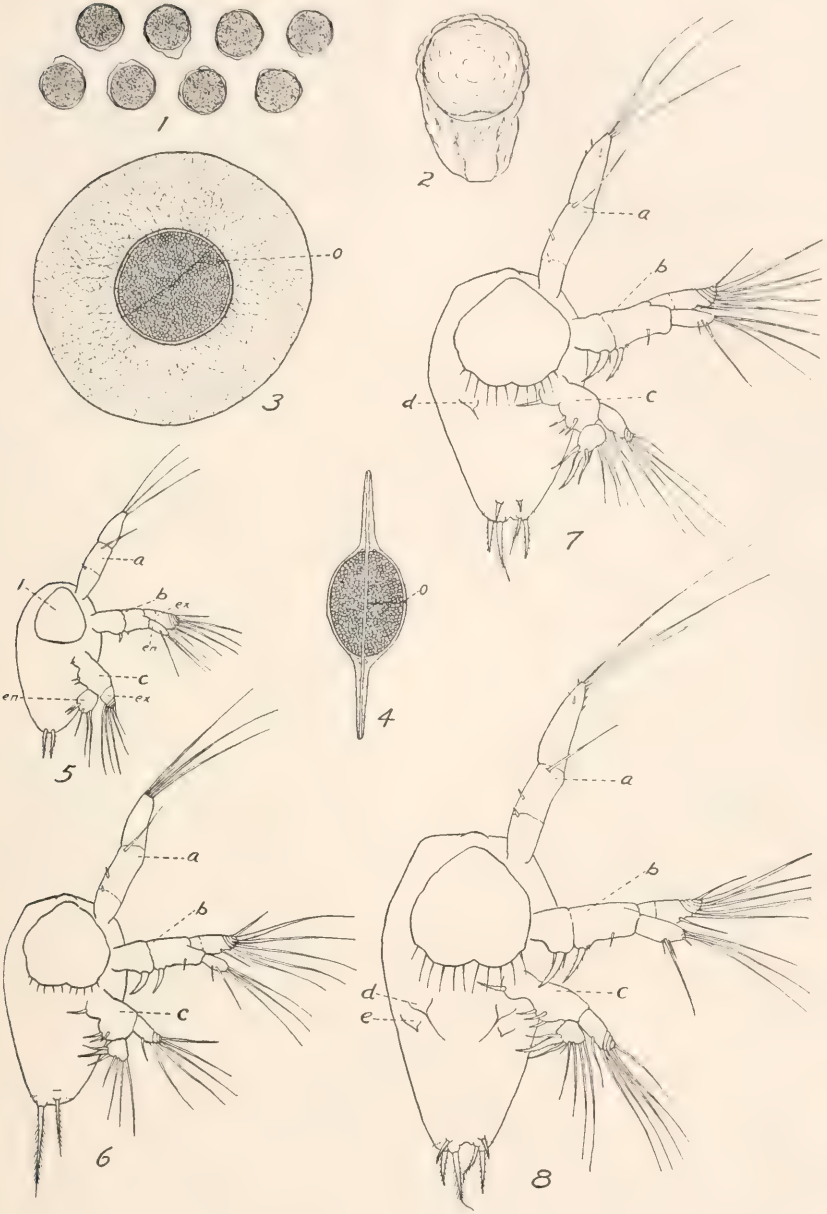
Abbreviations: *a*, first antenna; *b*, second antenna; *c*, mandible; *d*, first maxilla; *e*, second maxilla; *en*, endopodite; *ex*, exopodite; *f*, maxilliped; *g*, first feet; *h*, second feet; *i*, third feet; *l*, labrum; *o*, fissure.

EXPLANATION OF PLATE I

1. Eggs as deposited.
2. Egg showing formation of case.
3. Egg and completely formed case, viewed from above.
4. Egg and case viewed from side.
5. Nauplius, stage I.
6. Nauplius, stage II.
7. Nauplius, stage III.
8. Nauplius, stage IV.

PLATE I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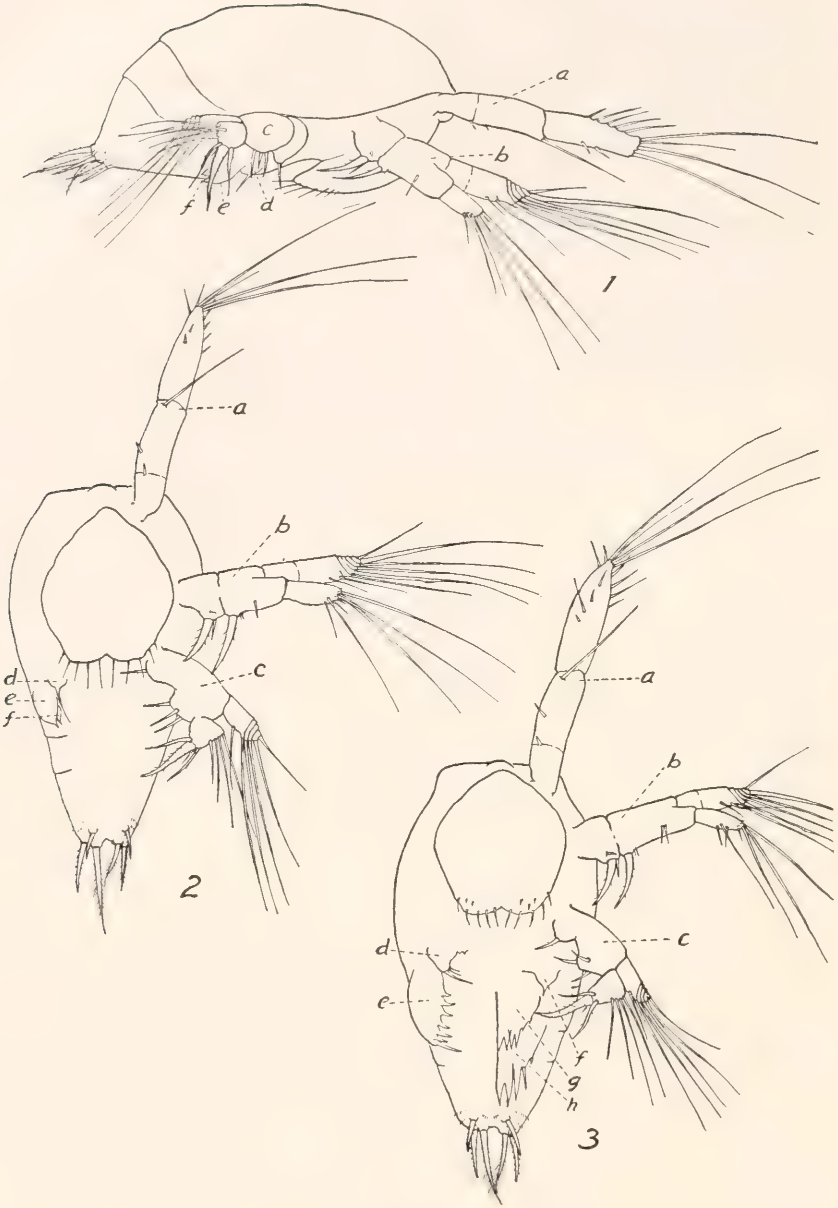
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PLATE II

1. Nauplius, stage V, viewed from side.
2. Nauplius, stage V.
3. Nauplius, stage VI.

being slightly ventral), and ventrally a pair of ventral hooks. Minute bristles present as in II.

First antenna. As in II, but with two short dorsal marginal bristles and one short ventral marginal bristle on the distal segment.

Second antenna. Coxopodite with one very strong masticatory hook and one small spine situated immediately beside it. Basipodite as in II. Endopodite as in II, but with one long and two short lateral bristles. Exopodite as in II.

Mandible. All parts as in II, but with an additional small bristle on the first segment of the endopodite.

First maxilla. Indicated only as a bud with a very weak bristle.

Stage IV (Plate I, Fig. 8)

Body 0.30 mm. long, the posterior end somewhat elongated, furcal armature as in III.

First antenna. As in III, but with four short dorsal marginal bristles and one short ventral marginal bristle on the distal segment.

Second antenna. Coxopodite and basipodite as in III. Endopodite as in III, but with the addition of one short terminal bristle. Exopodite as in III.

Mandible. All parts as in III, but with increased strength of spines.

First maxilla. As in III.

Second maxilla. Indicated only as a bud.

Stage V (Plate II, Figs. 1 and 2)

Body 0.32–0.34 mm. long, posterior end armed as in IV.

First antenna. As in IV, but with six short dorsal marginal and two short ventral marginal bristles on the distal segment.

Second antenna. Coxopodite, basipodite, and endopodite as in IV. Exopodite as in IV, but with one long and two shorter weak bristles on the second segment.

Mandible. All parts as in IV.

First maxilla. As in IV.

Second maxilla. Indicated as a bud.

Maxilliped. Indicated as a bud.

Stage VI (Plate II, Fig. 3)

Body 0.26 mm. long, posterior end armed as in V.

First antenna. As in V, but with six short dorsal marginal and four short ventral marginal bristles on the distal segment. Through the successive stages the distal segment has become relatively longer so it now about equals in length the other two combined.

Second antenna. Coxopodite as in V. Basipodite as in V, but with two short weak bristles placed side by side on the posterior margin near the outer end. Endopodite and exopodite as in V.

Mandible. As in V.

First maxilla. With three small unequal terminal bristles and two inconspicuous spines at its base.

Second maxilla. Rudimentary, with seven weakly chitinized bristles.

Maxilliped. Indicated only as a bud.

First and second feet. Rudimentary.

COPEPODID STAGES

Stage I (Plate III, Figs. 1 and 2)

Body 0.55 mm. long, thorax of four segments, urosome of one deeply constricted segment. Caudal rami symmetrical, each with three long terminal bristles, one short dorsal bristle, and two outer lateral spines. In life the first antennæ are noticeably directed upward as well as laterally and the urosome is also flexed strongly upward. The second antennæ are deep reddish-brown. This pigmentation may occur as isolated patches near the base of the appendages and is also found on the ventral side of the thorax. The coloration persists in the later stages but is less obvious in the second antennæ.

First antenna. Eight segments.

Second antenna. Coxopodite a short segment with no hook. Basipodite with one posteriorly directed bristle. Endopodite of two segments, the first being fused with the basipodite and bearing one distal bristle. The second segment bears at the tip five long bristles and on the inner margin a series of short fine bristles. Exopodite of four or five segments, the first short and with no bristles, the second long and bearing two long bristles, 3–4 very short with a total of four long bristles.

Mandible. Mandibular blade strong and provided with three well-defined teeth and one or two smaller teeth. The palp consists of a short

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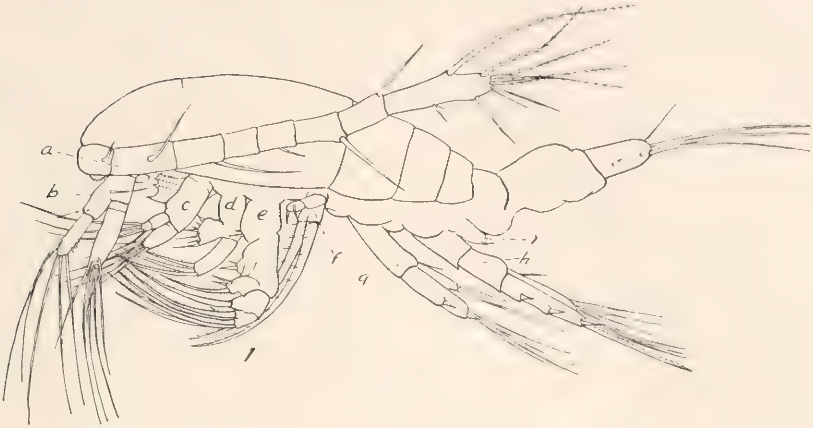
EXPLANATION OF PLATE III

1. Copepodid, stage I, viewed from side.
2. Copepodid, stage I, dorsal outline.
3. Urosome and caudal rami, stage II.
4. Urosome and caudal rami, stage III.
5. Urosome and caudal rami, stage IV.
6. Urosome and caudal rami, stage V. [Segments of thorax and urosome as numbered.]
7. Maxilliped, stage I.
8. Maxilliped, stage II.
9. Maxilliped, stage III.
10. Maxilliped, stage IV.
11. Maxilliped, stage V.

PLATE III



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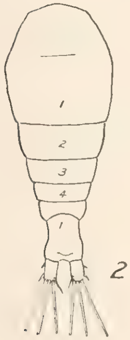

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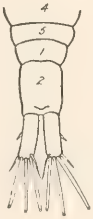

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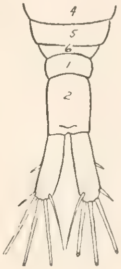

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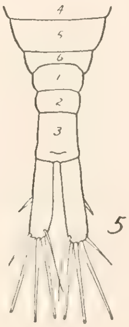

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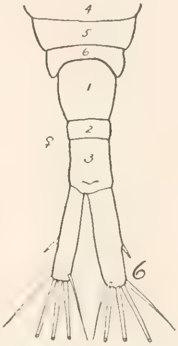

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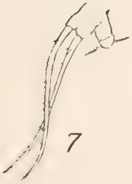

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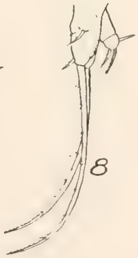

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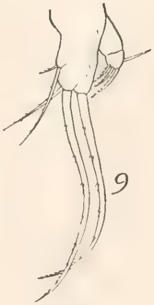

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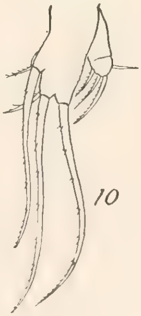

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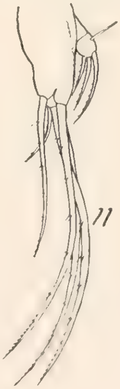

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coxopodite, long basipodite, and two short rami, the inner having two segments and bearing four long bristles at the end, the outer ramus bearing five long bristles.

First maxilla. There is a short rounded inner lobe armed with eight short bristles of unequal length, and a long outer segment bearing three long, strong, coarsely plumose bristles.

Second maxilla. Uniramose, very long and strong and composed of six or seven poorly defined wedge-shaped segments. The distal segments are armed with six long, strong coarsely plumose bristles, and one short coarsely plumose bristle. There are also six short bristles on the inner margin of the proximal segments. Two of these bristles are situated at the base of the first long bristle (Plate III, Fig. 1, *e*).

Maxilliped (Plate III, Fig. 7). First basal armed with two long, strong coarsely plumose bristles. Inner branch with one short plumose bristle directed anteriorly and one short smooth bristle directed posteriorly.

First feet (Plate IV, Fig. 1). Coxopodite and basipodite with no bristles. Endopodite unsegmented with seven bristles. Exopodite unsegmented with two outer spines and four bristles.

Second feet (Plate IV, Fig. 6). Coxopodite and basipodite with no bristles. Endopodite unsegmented with six bristles. Exopodite unsegmented with three outer spines, a terminal blade, and three inner bristles.

Third feet. Rudimentary.

Stage II

Body 0.74 mm. long, thorax of five segments, urosome of two segments, caudal rami symmetrical as in I (Plate III, Fig. 3).

First antenna. Eleven or twelve segments.

Second antenna. Coxopodite and basipodite as in I. Endopodite as in I, but with one short and four long terminal bristles. Exopodite as in I but with a total of only five bristles.

Mandible. As in I.

First and second maxilla. As in I.

Maxilliped (Plate III, Fig. 8). First basal with two long bristles as in I, and two very short bristles. Inner branch as in I, but with two plumose anteriorly directed bristles and one smooth posteriorly directed bristle.

First feet (Plate IV, Fig. 2). Coxopodite with one inner bristle. Basipodite with no bristle. Endopodite of two segments, the first with one inner bristle and the second with seven bristles. Exopodite of two segments, the terminal one with two outer spines and five end and inner bristles.

Second feet (Plate IV, Fig. 7). Coxopodite with one inner bristle. Basipodite with no bristle. Endopodite unsegmented, with eight bristles. Exopodite of two segments, the first with one outer spine; the second with two outer spines, a terminal blade, and four inner bristles.

Third feet (Plate IV, Fig. 11). Coxopodite and basipodite with no bristles. Endopodite unsegmented, with six bristles. Exopodite unsegmented, with three outer spines, a terminal blade, and three inner bristles.

Fourth feet. Rudimentary.

Stage III

Body 0.96 mm. long, thorax of six segments, urosome of two segments, caudal rami symmetrical as in II (Plate III, Fig. 4).

First antenna. Fourteen segments.

Second antenna. As in II, but with a group of minute inner bristles on the distal end of the first segment of the endopodite.

Mandible. As in II.

First maxilla. As in II.

Second maxilla. As in II, but with seven long, strong bristles and one short bristle on the distal segments and one small claw-like bristle on the basal segment.

Maxilliped (Plate III, Fig. 9). First basal with two long bristles as in II and with three short bristles. Inner branch as in II, but with three plumose anteriorly directed bristles and one smooth posteriorly directed bristle.

First feet (Plate IV, Fig. 3). Coxopodite with one inner bristle. Basipodite with no bristle. Endopodite of two segments, the first with one bristle and the second with eight bristles. Exopodite of two segments, the first with one small inner bristle and the second with two outer spines and five end and inner bristles.

Second feet (Plate IV, Fig. 8). Coxopodite with one inner bristle. Basipodite with no bristle. Endopodite unsegmented, with nine bristles. Exopodite of two segments, the first with one outer spine and the second with three outer spines, a terminal blade, and five inner bristles.

Third feet (Plate IV, Fig. 12). Coxopodite with one inner bristle. Basipodite with no bristle. Endopodite unsegmented, with nine bristles. Exopodite of two segments, the first with one outer spine; the second with two outer spines, a terminal blade, and four inner bristles.

Fourth feet (Plate IV, Fig. 15). Coxopodite and basipodite with no bristles. Endopodite unsegmented, with six bristles. Exopodite unsegmented, with three outer spines, a terminal blade, and three inner bristles.

Fifth feet. Rudimentary.

Stage IV

Body 1.11 mm. long, thorax of six segments, urosome of three segments in both male and female, caudal rami asymmetrical, the right ramus being slightly the longer (Plate III, Fig. 5).

First antenna. Sixteen segments.

Second antenna. As in III.

Mandible. As in III.

First and second maxilla. As in III.

Maxilliped (Plate II, Fig. 10). First basal with three long, strong bristles and two short bristles. Inner branch as in III.

First feet (Plate IV, Fig. 4). Coxopodite with one inner bristle. Basipodite with no bristle. Endopodite and exopodite as in III.

Second feet (Plate IV, Fig. 9). Coxopodite with one inner bristle. Basipodite with no bristle. Endopodite unsegmented, with ten bristles. Exopodite of two segments, the first with one outer spine and one inner bristle; the second with three outer spines, a terminal blade, and five inner bristles.

Third feet (Plate IV, Fig. 13). Coxopodite with one inner bristle. Basipodite with no bristle. Endopodite unsegmented, with eight bristles. Exopodite of two segments, the first with one outer spine; the second with three outer spines, a terminal blade, and five inner bristles.

Fourth feet (Plate IV, Fig. 16). Coxopodite with one inner bristle. Basipodite with no bristle. Endopodite unsegmented, with eight bristles. Exopodite of two segments, the first with one outer spine; the second with three outer spines, a terminal blade, and five inner bristles.

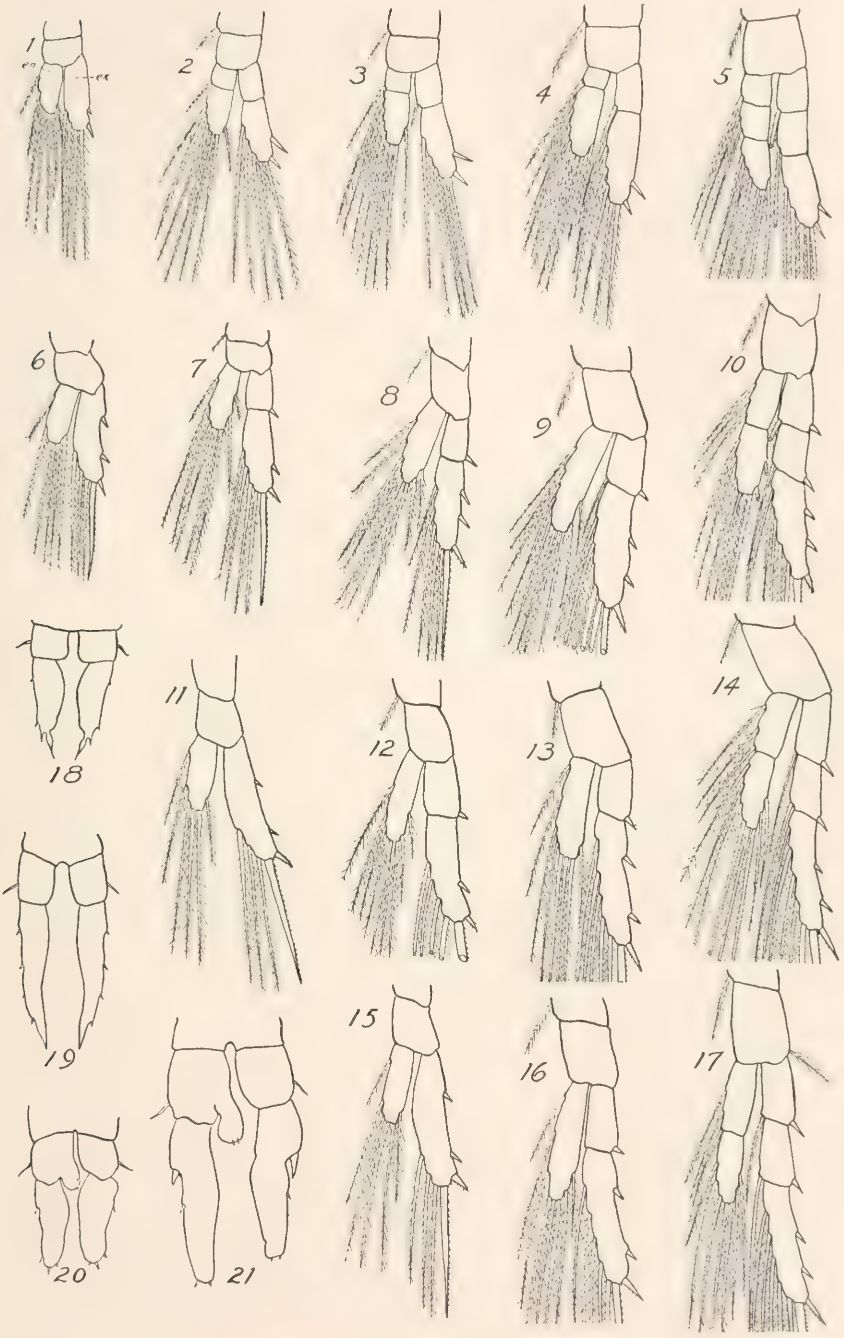
Fifth feet female (Plate IV, Fig. 18). Uniramose, symmetrical, of two segments, the first with one outer bristle, the second with two small outer marginal spines and one small and one larger terminal spine.

Fifth feet male (Plate IV, Fig. 20). Uniramose, asymmetrical, of two segments. The first segment of right foot with one short outer bristle and an inner thumb-like process bearing two small spines, the second segment with one small outer marginal spine and three terminal spines. Left foot as the right but wanting the thumb-like process.

EXPLANATION OF PLATE IV

1–5. Development of first feet, stages I–V.
6–10. Development of second feet, stages I–V.
11–14. Development of third feet, stages II–V.
15–17. Development of fourth feet, stages III–V.
18–19. Development of fifth feet, stages IV–V, female.
20–21. Development of fifth feet, stages IV–V, male.

PLATE IV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Stage V

Body 1.24–1.40 mm. long, thorax of six segments, urosome of three segments in female and four in male, caudal rami asymmetrical, the right being longer and wider than the left (Plate III, Fig. 6). Through the successive stages there has been an increase in the length of both rami in proportion to their width, and the posterior outer marginal spine has assumed the character of the terminal bristles.

First antenna. Eighteen segments.

Second antenna. As in IV.

Mandible. As in IV.

First and second maxilla. As in IV.

Maxilliped (Plate III, Fig. 11). First basal with three long, strong bristles, one medium long bristle, and one short bristle. Inner branch as in IV.

First feet (Plate IV, Fig. 5). Coxopodite with one inner bristle. Basipodite with no bristle. Endopodite of three segments, the first with one inner bristle, the second with two inner bristles, and the third with six bristles. Exopodite of three segments, the first and second with one inner bristle, the third with two outer spines and five end and inner bristles.

Second feet (Plate IV, Fig. 10). Coxopodite with one inner bristle. Basipodite with no bristle. Endopodite of two segments, the first with three inner bristles, the second with eight bristles. Exopodite of three segments, the first and second with one outer spine and one inner bristle; the third with three outer spines, a terminal blade, and five inner bristles.

Third feet (Plate IV, Fig. 14). As second feet in V.

Fourth feet (Plate IV, Fig. 17). Coxopodite with one inner bristle. Basipodite with one outer bristle. Endopodite of two segments, the first with three inner bristles, the second with seven bristles. Exopodite of three segments, the first and second with one outer spine and one inner bristle; the third with three outer spines, a terminal blade, and five inner bristles.

Fifth feet female (Plate IV, Fig. 19). Symmetrical, similar to IV, but the end segment more elongate and bearing four small outer marginal spines.

Fifth feet male (Plate IV, Fig. 21). Asymmetrical, similar to IV, but with one strong and one weak outer marginal spine and two weak terminal spines.

Stage VI. Adult

DISCUSSION

Tortanus discaudatus is a predacious species with specialized mouth parts for use in capturing and holding its prey, which consists largely of copepod larvæ and other smaller copepods. This carnivorous habit is also present in the larval stages, where the recognizable food is made up of tintinnids, mainly *Tintinnopsis* and *Stenosomella,* two genera abundant in this locality. Nearly 50 per cent of the larvæ taken from the plankton contained loricæ of these ciliates during the season when they were numerous.

The long first antennæ, the large masticatory hooks on the second antennæ, the heavy hook on the gnathobase of the mandible, and the arrangement of the armature on the posterior end of the body serve to distinguish the nauplius larva of *Tortanus discaudatus* from that of any other described copepod larva. The closest resemblance appears to be to the larvæ of *Acartia longiremis* and *Acartia bifilosa.* But this is a rather remote resemblance which is most noticeable in the shape and bristles of the mandibles and of the first antennæ. Other characteristics common to these species are the fusion of the first and second segments of the first antenna and the first and second segments of the exopodite of the second antenna. The furcal armature also recalls that of *Acartia.* Some resemblance was to be expected, for *Tortanus* and *Acartia* are remotely allied genera.

Table I gives the characters most useful in the identification of the nauplius stages of *Tortanus discaudatus.*

After the first nauplius stage the mandibles show but little change other than the strengthening of the spines and bristles and have therefore been omitted from the table. Size is perhaps the one most useful character, though the variations are sufficient to cause some confusion between two successive stages. The first, second, and sixth stages are comparatively easy to determine, while the third, fourth, and fifth occasion some difficulty.

In the fifth copepodid stage the animal has assumed practically all the adult structural peculiarities, and the changes that follow this stage relate mainly to sexual maturation and are mostly a matter of change in relative proportions. The moult from the fourth to the fifth copepodid stage is particularly significant in the development of the swimming feet. In the earlier stages each branch of each foot begins with a single segment and in the following moult the exopodite (and also the endopodite of the first feet) undergoes one division and remains thus until the change to the fifth stage, when each branch of all the feet (excepting the fifth pair) again undergoes one division, giving the total number of segments found in the adult. The fifth feet, especially in the male, are subject to considerable change on entering the sixth or adult stage, but it is to be noted that the right foot in the male did not become three-segmented, as described for the adult (*Corynura bum-*

TABLE I

Identification Table for Nauplius Stages of Tortanus discaudatus

		I	II	III	IV	V	VI
Length of body (in mm.)		0.12–0.14	0.185–0.205	0.23–0.26	0.30	0.32–0.34	0.36
First Antenna	Proximal segment	1 short bristle 1 long bristle	2 short bristles 1 long bristle	As in II	As III	As IV	As V
	Distal segment	3 terminal bristles	4 terminal bristles	As II but 2 dorsal and 1 ventral marginal bristle	As III but 4 dorsal and 1 ventral marginal bristle	As IV but 6 dorsal and 2 ventral marginal bristles	As V but 6 dorsal and 3 ventral marginal bristles
Second Antenna	Endopodite	2 terminal and 1 lateral bristle	3 terminal, 2 lateral bristles	3 terminal, 3 lateral bristles	3 long, 1 short terminal, 3 lateral bristles	As IV	As V
	Exopodite	6 bristles	7 bristles	7 bristles	7 bristles	8 bristles	8 bristles
Caudal spines		2 identical	2, the right longer, heavier	6 uneven length	6 uneven	6 uneven	6 uneven
First maxilla		0	0	Bud	Bud	Bud	Rudimentary
Second maxilla		0	0	0	Bud	Bud	Rudimentary
Maxilliped		0	0	0	0	Bud	Bud
First feet		0	0	0	0	0	Rudimentary
Second Feet		0	0	0	0	0	Rudimentary

pusii) by Wheeler (1899, p. 185, Fig. 21, *f*) but remains as figured by Thompson and Scott (1897, Plate 7, Fig. 2). The sexes are first distinguishable in the fourth stage by the structure of the fifth feet.

Following the moult to the sixth stage, a fifth segment is added to the urosome of the male, and in both sexes the urosome and caudal rami become suddenly much more asymmetrical and the lateral spine of the right caudal ramus becomes far more prominent. This asymmetry was indicated in earlier stages but remained latent and in this respect is similar to the characters accentuated by sexual maturation, with which it is doubtless closely associated. The asymmetry is most striking in actively spawning specimens and it has been noted that in the female the spermatophore is attached to the right lateral spine as well as to the genital segment. Asymmetry is first introduced with the second nauplius stage and persists through the following nauplius stages. Perfect symmetry again appears in the urosome and caudal rami of the first copepodid and persists also through the second and third copepodid stages. Following this there is a gradual small increase in asymmetry till the last sudden increase coming with the adult stage as mentioned

TABLE II

Identification Table for Copepodid Stages of Tortanus discaudatus

	I	II	III	IV	V
Length of body (in mm.)	0.55	0.74	0.96	1.11	1.24–1.4
First antenna	8 segments	11 or 12 segments	14 segments	16 segments	18 segments
Maxilliped, first basal	2 long bristles	2 long and 3 very short bristles	2 long and 3 short bristles	3 long and 2 short bristles	3 long, 1 medium, and 1 short bristle
Feet present	1st and 2nd (3rd as buds)	1st, 2nd and 3rd (4th as buds)	1st, 2nd, 3rd and 4th (5th as buds)	1st, 2nd, 3rd, 4th, and 5th	As in IV
Thorax	4 segments	5 segments	6 segments	6 segments	6 segments
Urosome	1 segment	2 segments	2 segments	3 segments	♀ 3 seg. ♂ 4 seg.
Caudal rami	Symmetrical	Symmetrical	Symmetrical	Right ramus slightly the longer	Right ramus the longer and broader

above. Plate III, Fig. 6, is for the female, but the male also shows a gradual change to asymmetry after the third copepodid stage and in the fifth stage the caudal rami are about as in the female but the lateral spine on the right caudal ramus is noticeably larger and there is a very slight indication of the pointed process found in the right distal corner of the first abdominal segment of the adult.

It will be noted that the appendages which change least during the successive moults of the copepodid stages are the second antennæ, the mandibles, and the first and second maxillæ. These are therefore of little use in determining the various stages.

Table II gives briefly some of the more salient features useful in identification of the copepodid stages.

SUMMARY

1. Identification of the pelagic eggs of *Tortanus discaudatus* is established by hatching larvæ from eggs occurring in the plankton and also from eggs definitely known to have been spawned by the species in experimental cultures.

2. A description is given of the development of the specialized flotation case containing the egg.

3. The nauplius stages I–VI are described and figured.

4. The copepodid stages I–V are described and figures given.

5. The nauplius larva has a remote resemblance to the nauplius larvæ of *Acartia longiremis* and *Acartia bifilosa*.

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Vol. LXVII, No. 2 October, 1934

THE

BIOLOGICAL BULLETIN

PUBLISHED BY THE MARINE BIOLOGICAL LABORATORY

OBSERVATIONS ON THE BINARY FISSION OF FOUR SPECIES OF COMMON FREE-LIVING CILIATES, WITH SPECIAL REFERENCE TO THE MACRONUCLEAR CHROMATIN

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In spite of the many and interesting papers that have been published on the behavior of the various cell elements of the Ciliata during the regular process of binary fission, our knowledge of the cytological details of the process is limited to a surprisingly small group. With a few notable exceptions, the observations have been made on the very large forms or upon the bizarre forms infrequently encountered. Common forms, such as *Colpidium* and *Glaucoma*, which have been repeatedly used for physiological experiments, have suffered neglect as far as their divisional process is concerned. It is the purpose of this paper to report the results of our investigations of the fission macronuclei of four species of cosmopolitan fresh-water ciliates: *Urocentrum turbo, Colpidium campylum, Colpidium colpoda,* and *Glaucoma scintillans.* While the micronuclei of all four species were observed in all phases of mitosis, we will treat these observations only as incidental to the main problem.

Material and Methods

Urocentrum turbo and *Colpidium colpoda* were collected from Heneage Pond near Hanover, New Hampshire, while the *Colpidium campylum* and *Glaucoma scintillans* used for this investigation were collected from the lake in Van Cortlandt Park, New York City.

Urocentrum proved to be the least adaptable form and grew poorly in pure culture. Enormous numbers were obtained, however, in mixed cultures. The procedure was as follows. Wild material was brought into the laboratory and placed in a battery jar and allowed to stand for two or three days. A small piece of lettuce leaf was then floated on the surface of the culture and the whole allowed to stand from two to three weeks. Examinations were made from time to time to determine the richness of the culture, and when the *Urocentrum* became very abundant material was removed from near the surface, centrifuged for concentration, and fixed in bulk.

Colpidium colpoda was grown in mixed cultures in great abundance, and quantities of material from the same source as that used for *Urocentrum* were prepared for study. Pure cultures were easily obtained by inoculating a single individual into a boiled rye medium, prepared after the manner of Sonneborn (1932). Hay tea, hay-wheat, and rice were also used and proved to be quite satisfactory for this ciliate.

Colpidium campylum and *Glaucoma scintillans* were grown in pure culture by inoculating previously prepared media with isolated individuals. The rye medium of Sonneborn (1932) proved very good for *Colpidium campylum,* while a boiled rice medium yielded flourishing cultures of *Glaucoma scintillans.*

In all cases care was taken to prepare for cytological study only those ciliates from new and flourishing cultures. Old cultures, in which the conditions had ceased to be favorable for rapid division, were excluded. Because of this it seems probable that the details we are giving represent the normal activity of healthy organisms.

Employing the centrifuge for concentration, bulk material was fixed, stained, dehydrated, cleared, and mounted. In some cases, after staining, the material was dehydrated to 70 per cent alcohol and then dropped on to albuminized cover-glasses, following the method outlined by Summers (1934). Either method gave uniform and excellent results.

For the bulk material the best results were obtained by fixing in warm Schaudinn's fluid or in Perenyi's fluid. After fixation the material was stained in Heidenhain's hæmatoxylin, Borax carmine, or by the Feulgen nucleic acid reaction. The Feulgen material was counterstained in light green or cyanin plus picric acid.

In studying the macronuclear activity of *Colpidium campylum, C. colpoda,* and *Glaucoma scintillans* immediately following fission, individual fixing and staining was employed. Ciliates in some obvious stage of binary fission were isolated and allowed to divide. At varying intervals after the two daughters had separated they were fixed on cover-glasses by the drop method, stained, and mounted. For permanent preparations of these daughters Schaudinn's fluid followed by Heidenhain's hæmatoxylin or the Feulgen nucleic acid reaction was used. For temporary mounts good results were obtained with aceto-carmine.

For identification of the species studied we found the silver method of Klein (1926*a*, 1926*b*) to be of great value. This method, together with the relief staining technique of Bresslau (1922) and the standard hæmatoxylin preparations, gave results that checked precisely with the previously recorded descriptions of the four species used (Bresslau, 1922; Klein, 1926*b*, 1928; Kahl, 1931).

In the case of *Colpidium campylum* it was found possible to watch the whole process of macronuclear fission in the living organism. Under the oil immersion lens the macronuclei can be seen quite clearly and the activity followed from the beginning of the divisional process until the daughter ciliates are completely reorganized. In this manner we have provided direct evidence for the peculiar process of chromatin elimination from the macronucleus during division which substantiates in every detail the circumstantial evidence obtained from fixed and stained material.

Observations

Urocentrum turbo

The trophozoite of this species is too well known from the numerous descriptive works to warrant a lengthy discussion of its general morphology. The macronucleus is located in the posterior fourth of the body. It is in the form of a *C*, the ends of which are club-shaped and connected to each other by an attenuated strand. The *C*-shaped macronucleus lies with its greatest diameter perpendicular to the long axis of the cell (Fig. 1, *A*) with the open part directed toward the oral side, somewhat posterior to the mouth (Fig. 1, *B*). It partially encircles the posteriorly located contractile vacuole. The chromatin seems to be concentrated in the two club-shaped ends of the macronucleus and these ends therefore stain quite intensely. The attenuated strand connecting the two ends of the *C* stains very faintly and in some cases it is observed with difficulty. The micronucleus lies just posterior to the attenuated strand and slightly toward the aboral periphery of the cell (Fig. 1, *A* and *B*). It is small and spherical and stains very feebly with all of the nuclear dyes. The chromatin appears to be homogeneous. Both the macronucleus and the micronucleus of *Urocentrum* shrink somewhat upon fixation. This shrinkage results in a clear region between the chromatin and the cytoplasm and is quite characteristic of all stages.

The first evidence of the approach of binary fission is the swelling of the micronucleus to approximately twice its original diameter. The chromatin remains homogeneous and stains even more faintly than before. Concomitant with the micronuclear swelling the macronucleus thickens and shortens, and the two club-shaped ends draw together. The attenuated strand disappears and the macronucleus becomes ovoid (Fig. 1, *C* and *D*). At this time the nuclei change their orientation in relation to the cell, the division macronucleus moving into a position so that its long axis parallels the long axis of the ciliate (Fig. 1, *E*). The micronucleus proceeds to the metaphase and a large spindle is formed

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upon which are arranged numerous short chromosomes (Fig. 1, *E*). Because of the faint staining quality of the micronuclear chromatin at all times, and especially during mitosis, we have been unable to observe the fine details of this process. In a few favorable preparations we were able to observe the nature of the chromosomes on the spindles but in no case were we able to make chromosomal counts.

During the anaphase of the micronucleus the macronuclear chromatin becomes differentiated into regions. A band of deeply staining chromatin, separated from the macronuclear ends by two clear zones, makes its appearance in the mid-region (Fig. 1, *F*). By this time the ciliate shows marked elongation and the two daughter cytostomes are quite distinct. As the daughter micronuclei separate at the telophase the macronucleus elongates and starts to constrict in the region of the central, deeply staining band (Fig. 1, *G*). The constriction continues and the central chromatin becomes concentrated into a small mass of intensely staining material, while the clear zones bounding the central mass become less conspicuous. These clear zones become the two planes of fission which finally pinch off the central mass from the daughter macronuclei (Fig. 1, *H* and *I*). The central mass of intensely staining chromatin is cast into the cytoplasm where it assumes a spherical form. As the daughter macronuclei move apart and prepare to reorganize, the extruded chromatin is moved about in the cytoplasm

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FIG. 1. *Urocentrum turbo.* Camera lucida drawings. × 567.

A. Side view of a resting individual, showing the position of the nuclei. Schaudinn's: Feulgen.

B. Polar view of a resting individual. Schaudinn's: Feulgen.

C. Polar view of an individual in the early stages of binary fission. The micronucleus is swollen and the attenuated strand has thickened. Schaudinn's: Feulgen.

D. Later stage than *C*. Schaudinn's: Feulgen.

E. The fission nuclei have become oriented with their long axes parallel to the long axis of the cell. The micronucleus is in full metaphase and there are two cytostomes present. Perenyi's: Carmine.

F. Slightly later stage. The micronucleus is in the anaphase and the central, deeply-staining chromatin has become differentiated in the macronucleus. Perenyi's: Carmine.

G. The micronucleus has divided and the macronucleus has started to constrict. Schaudinn's: Feulgen.

H. The daughter micronuclei have moved apart and the macronucleus has further constricted. The central chromatin has contracted between the daughter halves of the macronucleus. Schaudinn's: Feulgen.

I. The daughter macronuclei are pulling apart, leaving the central chromatin mass at the plane of fission. Schaudinn's: Feulgen.

J. The central chromatin has drifted toward the cell periphery and is starting to disintegrate in the cytoplasm. The posterior daughter macronucleus is starting to draw out into the *C*-shape of the resting stage. Schaudinn's: Feulgen.

K. The central chromatin has been completely absorbed. The daughter macronuclei are both taking on the *C*-shape characteristic of the resting stage. Schaudinn's: Feulgen.

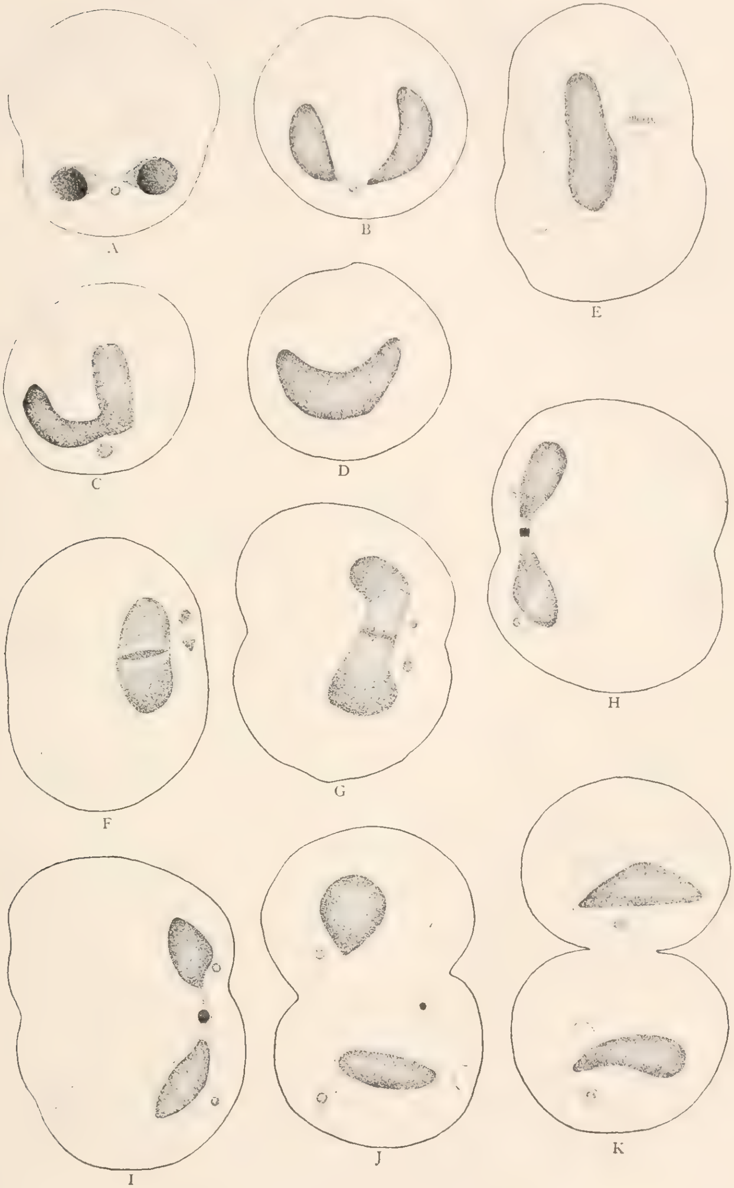
A
B
E
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FIG. 1

near the fission plane (Fig. 1, *J*). It becomes smaller and smaller until it ultimately disappears from view (Fig. 1, *K*), usually some time prior to the separation of the daughter ciliates. Reorganization of the nuclear apparatus in each daughter ciliate is far advanced before complete separation occurs. The daughter macronucleus of the posterior ciliate is usually a little in advance of that of the anterior. Eventually they both elongate in a plane perpendicular to the long axis of the division nuclei and the cell as a whole and assume the characteristic *C* shape (Fig. 1, *K*).

We have encountered a small percentage of *Urocentrum* with two macronuclei. In the resting stage it is usually difficult to decide whether or not these forms are really bi-macronucleate or normal forms with an attenuated strand so thin as to escape detection. During the advance division stages, however, they can be identified with ease. Each of the macronuclei behaves precisely as the single macronucleus of the normal form does. The formation and casting out of the deeply staining chromatin from the central region occurs in both macronuclei simultaneously. Indeed, the activity of both macronuclei has been found to be exactly synchronous in every respect (Fig. 2, *A* and *B*). As to the reorganization of the daughter macronuclei, we cannot say. Whether they remain separate and result in a bi-macronucleate race or whether they become joined to form a single macronucleus is an interesting question, but our material does not afford an answer at this time. We believe it probable, however, that the condition may be temporary and may have resulted from the rupturing of the slender central strand of the normal macronucleus sometime prior to the divisional activity.

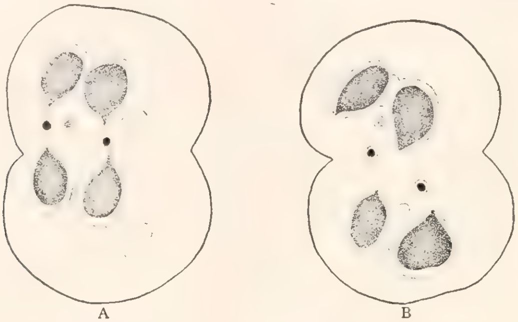

FIG. 2. *Urocentrum turbo.* Division of individuals with a double macronucleus. Camera lucida drawings. × 567.

A. Late division stage in which the daughter macronuclei are still connected with the central chromatin masses. Schaudinn's: Feulgen.

B. Later stage. The central chromatin masses have been cast into the cytoplasm. Schaudinn's: Feulgen.

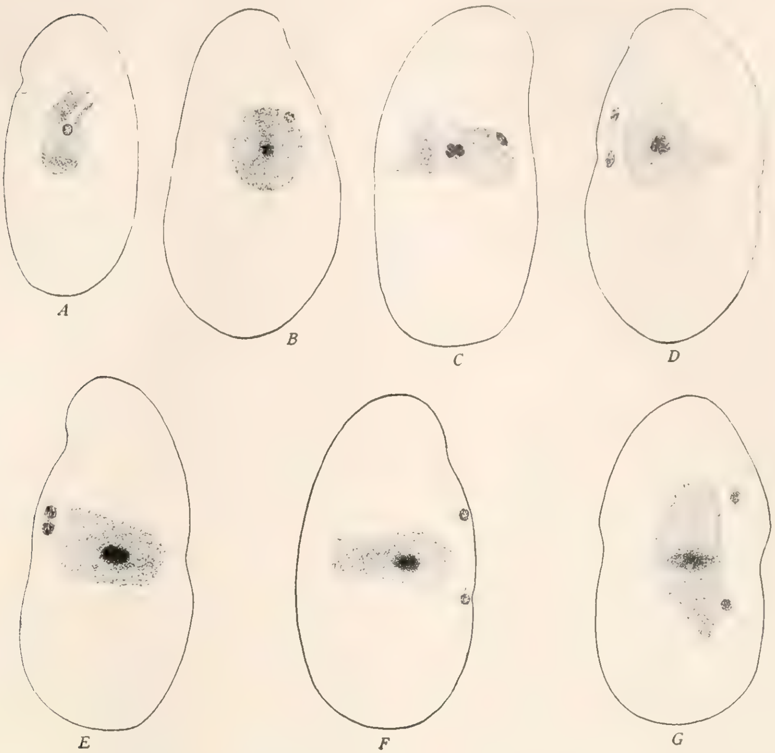

FIG. 3. *Colpidium campylum.* Camera lucida drawings from material fixed in Schaudinn's fluid and stained with the Feulgen nucleic acid reaction. × 567.

A. Resting individual. The micronucleus is seen in a depression of the irregular macronucleus.

B. The micronucleus has swollen slightly and has emerged to the periphery of the macronucleus. The central chromatin is being differentiated in the center of the swollen macronucleus.

C. The micronucleus is in late prophase. The macronucleus has elongated at right angles to the long axis of the cell. The central chromatin appears to be composed of a number of small spheres. The cytostome of the posterior daughter ciliate is visible.

D. Telophase of the micronucleus. The central chromatin has enlarged.

E. Further enlargement of the central chromatin mass. In this individual the micronucleus is in the anaphase.

F. Daughter micronuclei separating. The central chromatin mass is starting to elongate at right angles to the long axis of the cell.

G. Anterio-posterior elongation of the macronucleus has taken place, resulting in the diamond-shaped appearance of the macronucleus. The central chromatin has become spread out across the middle.

Colpidium campylum

The macronucleus of *Colpidium campylum*, during the resting stage, is located in the anterior half of the body. It is irregular in outline and usually somewhat elongate in the direction of the long axis of the cell. The micronucleus is spherical and deeply staining and is situated in a groove or depression in the macronucleus (Fig. 3, *A*). Both the chromatin of the macronucleus and that of the micronucleus is very basophilic, the former disposed in fine granules while the latter forms a dense reticulum.

The first indication of the divisional process is a swelling and rounding up of the macronucleus. The micronucleus also swells somewhat and begins to emerge from its resting position. The prophase of mitosis is usually accomplished by the time the micronucleus has moved away from the macronucleus. By the time the two nuclei have drawn completely apart the micronuclear chromosomes have formed on the metaphase plate. The chromosomes are fine, thready structures and do not resemble in any way the spheres described by Ivanić (1928) for *Colpidium colpoda.* We have been unable to make chromosomal counts due to their small size and closely packed condition. The anaphase and telophase appear to be quite regular (Fig. 3, *D* and *E*) and the daughter micronuclei separate and become condensed (Fig. 3, *F* and *G*).

As the macronucleus rounds up, the chromatin begins to show evidence of differentiating into regions. A small sphere of intensely staining chromatin forms in the center. The collection of chromatin is usually a single mass (Fig. 3, *B*) but occasionally appears to be made up of an aggregate of smaller spheres (Fig. 3, *C*). Migration of the macronucleus now takes place until it comes to occupy a position in the mid-region of the elongating cell. The central mass of chromatin increases in size and staining capacity (Fig. 3, *D*). When the central mass has reached its maximum size the macronucleus elongates at right angles to the long axis of the cell (Fig. 3, *E* and *F*), until, in some cases, it stretches nearly the width of the body of the ciliate. After this cross elongation the macronucleus begins to pull out in a direction parallel to the long axis of the cell body, resulting in a diamond-shaped configuration. The deeply-staining central mass becomes spread out across the central region of the diamond-shaped macronucleus (Fig. 3, *G*). Further anterio-posterior elongation results in a peculiarly shaped nucleus, drawn out to points at each end and swollen in the mid-region. This figure is quite characteristic for this species (Fig. 4, *A*).

Further macronuclear elongation is followed by constriction in two planes, one on either side of the central mass of chromatin, but, instead of this process proceeding to completion as was the case in *Urocentrum turbo,* a third constriction makes its appearance. This plane divides the central mass into two, often very irregular and uneven, masses. This third plane becomes the actual plane of fission between

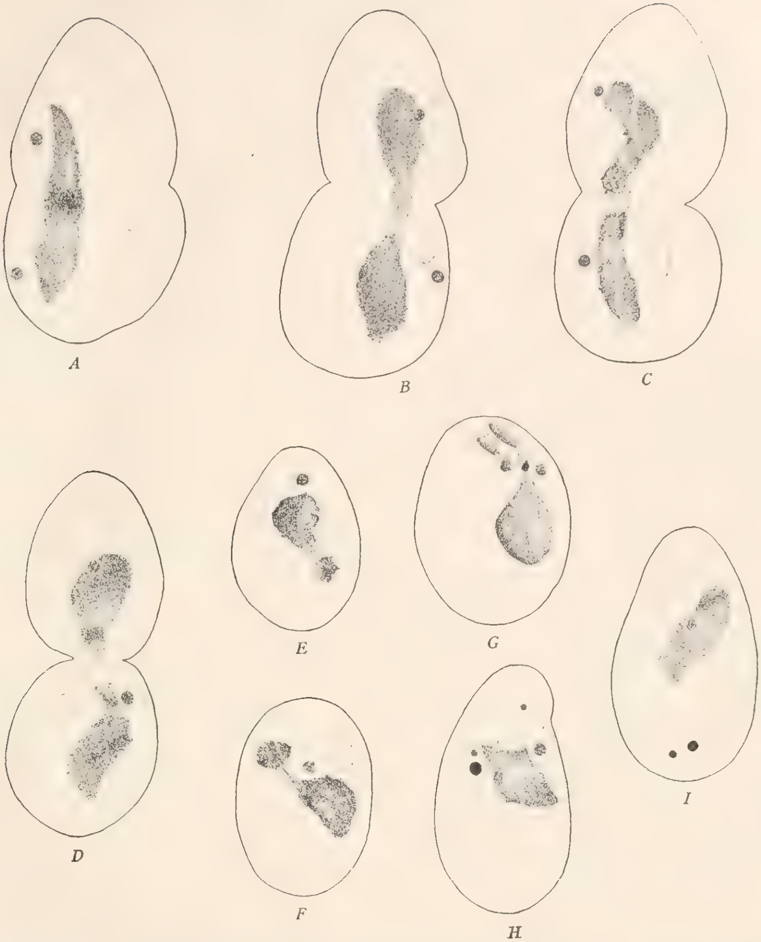

Fig. 4. *Colpidium campylum.* Camera lucida drawings from material fixed in Schaudinn's fluid and stained with the Feulgen nucleic acid reaction. × 567.

A. Macronucleus elongate. Constrictions are starting on either side of the central chromatin mass.

B. The central chromatin mass has divided and the macronucleus has become constricted between the two halves.

C. The chromatin of the central mass has become evacuolated and is only slightly different in staining capacity from that of the rest of the daughter macronuclei.

D. The daughter macronuclei have nearly separated. Plasmotomy is well under way.

E. An anterior daughter ciliate after division. The central chromatin is budding off from the macronucleus.

F. A posterior daughter ciliate in the same stage as *E.*

G. Fragmentation of the central chromatin bud. The micronucleus is seen to the right of the macronucleus.

H. The bud fragments have become deeply staining spheres and are drifting in the cytoplasm.

I. The nuclei have reorganized. In the posterior endoplasm two spheres of central chromatin are seen. These spheres represent the disintegrating products of the buds.

the daughter macronuclei while the previous constrictions distal to the central chromatin remain as relatively clear bands separating the chromatin of the daughter macronuclei from that of the central mass (Fig. 4, *B*). The cleavage of the central mass often results in two vacuolated halves in which the chromatin appears to have undergone a process of severe agitation (Fig. 4, *C*). Final separation of the daughter macronuclei occurs shortly before the daughter ciliates pull apart (Fig. 4, *D*), each daughter macronucleus retaining half of the chromatin that formed the central mass. In a great many cases the daughter macronuclei round up to such an extent that the identity of the central chromatin is lost in the chromatin of the macronucleus. In others the difference of staining capacity between the two is more marked.

In no case have we found any indication of the extrusion of chromatin into the cytoplasm during the actual cell fission in *Colpidium campylum*. The peculiar macronuclear phenomenon that ensues after plasmotomy must be followed in the single daughter ciliates.

In the daughter ciliates, shortly after their separation, each macronucleus undergoes a second elongation and takes on the appearance of a tear-drop, with the pointed end toward the original fission plane. The small, pointed end swells into a bud which proceeds to pinch off. This bud appears to be formed from the central chromatin which originally occupied the space between the fission nuclei. In most cases the bud, which is usually quite large (Fig. 4, *E* and *F*), is broken away from the macronucleus in a single piece but occasionally it fragments as it is being given off (Fig. 4, *G*). The fragments round up in the cytoplasm (Fig. 4, *H*) and are carried to the posterior region of the cell. They become very intensely staining and homogeneous, decrease gradually in size, and finally disappear from view. By this time the ciliate is growing and the nuclear apparatus is taking on the normal trophic appearance (Fig. 4, *I*).

The above process was followed in life from the start of the divisional activity until the daughter ciliates were completely reorganized, as was mentioned in the early pages of this account. It was found possible to observe the casting out of the buds of chromatin, the subsequent rounding up of the fragments of the buds and the drifting of these fragments to the posterior endoplasm of the cell. Here they could be seen for some time as refractile spheres but it was impossible to keep the daughter ciliates alive for a long enough time to observe their complete absorption into the cytoplasm.

It is evident from the above description that the budding process from the macronuclei of young daughter ciliates of *Colpidium campylum* represents a process of chromatin elimination similar to that found in *Urocentrum turbo*. The actual elimination is here delayed until after plasmotomy and the amount given off is proportionately many times greater.

Colpidium colpoda

The general activity of the nuclei of *Colpidium colpoda* in all phases of its divisional process is very similar to that of the preceding species. There are, however, a few differences that will be brought out in the following brief description.

The resting nuclei differ from those of *Colpidium campylum* in that the macronucleus is spherical and proportionately larger and is rarely found in contact with the micronucleus. The latter is typically situated near the periphery of the cell in the region of the cytostome. The macronuclear chromatin is disposed in regular, closely packed granules or less often in larger aggregates of granules while the micronuclear chromatin is arranged in a dense reticulum. The outlines of both nuclei tend to be much more regular than those of *C. campylum*.

The mitotic phases of the micronucleus follow the type seen in *Colpidium campylum*, with thready chromosomes forming on the metaphase plate. Here again they are too fine and closely packed to permit a count. Although we have examined countless spindles, we have never observed the regular spherules that were described and figured by Ivanić (1928) as the typical mitotic chromosomes of this species.

The appearance of the central ball of deeply-staining chromatin within the macronucleus occurs during the prophase of the micronucleus. It increases in size and staining capacity as the macronucleus migrates to the center of the cell (Fig. 5, *A*). The macronucleus never elongates at right angles to the longitudinal axis of the cell to the extent found in *Colpidium campylum*, but begins its anterio-posterior elongation almost as soon as the central position is attained. The central ball of deeply-staining chromatin spreads out to form a band across the mid-region of the elongating macronucleus, forming a characteristic bulge in the region of the fission plane. The deeply-staining central chromatin is marked off from the two ends of the macronucleus by two light bands, as if the chromatin were less concentrated in these regions (Fig. 5, *B*).

As constriction proceeds in the central region, the deeply-staining band contracts and becomes divided into two approximately equal halves. There is little if any indication of the vacuolation that occurs in *Colpidium campylum*, the halves remaining evenly granular and regular in outline (Fig. 5, *C*). As the daughter macronuclei pull apart, the two halves of the central mass become somewhat rounded. They retain their identity, however, and do not mix with the chromatin of the daughter macronuclei.

In the majority of cases the daughter ciliates separate, as in the preceding species, before any fragmentation on the part of the central chromatin has taken place. In a number of forms, however, we have observed small portions of the central chromatin fragmenting into the cytoplasm before plasmotomy was completed (Fig. 5, *D*). These fragments vary in size and number and are usually irregular. They appear to disintegrate rapidly in the cytoplasm without the characteristic contraction of the later disintegration spheres.

As the daughter ciliates separate or shortly thereafter, the central chromatin begins to contract and to form a bud-like projection from the macronucleus. Figure 5, *E*, is a camera lucida drawing of the two daughter organisms of a pair just at separation. The protoplasmic connection between the two is still visible, and the buds are pinching off from their respective daughter macronuclei. The chromatin of the buds continues to contract and becomes more and more basophilic (Fig. 5, *F*) until it finally separates from the macronucleus. It then fragments into a varying number of spheres that are slowly swept into the posterior endoplasm of the cell, where they are absorbed into the cytoplasm.

The amount of chromatin eliminated varies in different individuals. In some a relatively small bud is formed which breaks up into two or three disintegration spheres, while in others the bud is extremely large, resulting in the formation of from six to eight spheres. Figure 5, *G*, represents an organism of the latter type. The cell has undergone considerable growth and the nuclei appear to be completely reorganized, but the disintegrating spheres are still very much in evidence. In a few cases we have observed ciliates in the initial stages of fission in which a

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FIG. 5. *Colpidium colpoda*. Camera lucida drawings. × 567.

A. Early division stage. The micronucleus is in the late prophase and the central chromatin is at its maximum size and concentration. Schaudinn's: Feulgen.

B. Elongation and constriction of the macronucleus. The central chromatin forms the deeply staining band in the center. Schaudinn's: Feulgen.

C. Further constriction of the macronucleus. The central chromatin mass has divided. Schaudinn's: Feulgen.

D. Complete separation of the daughter macronuclei and the halves of the central chromatin mass. In this specimen three small and irregular fragments have broken away from the central chromatin and lie in the cytoplasm near the fission plane. Schaudinn's: Feulgen.

E. Two daughter ciliates nearly separated. The central chromatin is being budded off into the cytoplasm. Perenyi's: Borax Carmine.

F. Daughter ciliate shortly after division. The bud of central chromatin is very contracted. Schaudinn's: Feulgen.

G. Daughter ciliate. The chromatin of the bud has fragmented into spheres which have rounded up and drifted away from the macronucleus. The nuclei appear to be completely reorganized. This individual was extremely large, indicating that a considerable length of time had elapsed since division. Perenyi's: Borax Carmine.

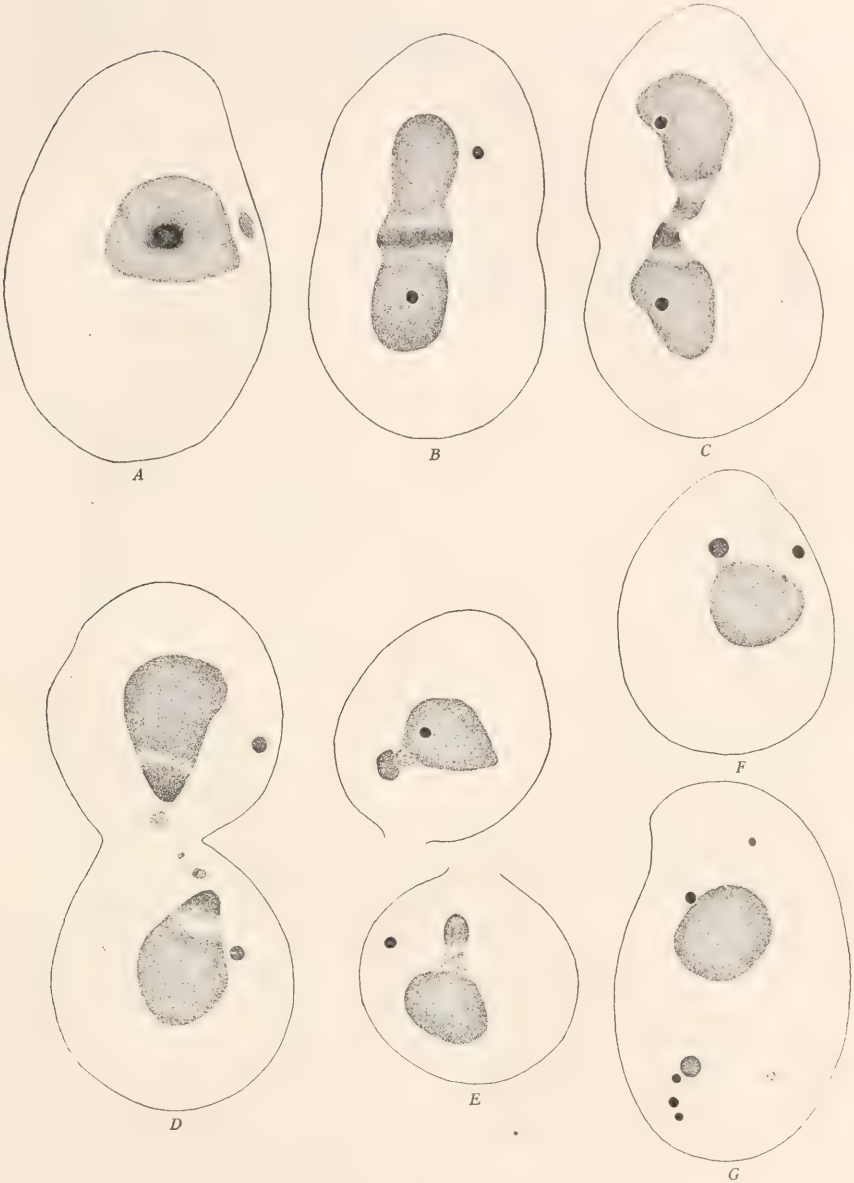

FIG. 5

few persisting spheres of old extrusion chromatin could be seen. This would seem to indicate that the complete reorganization of the cell was not dependent upon the absorption of the chromatin into the cytoplasm but rather upon the elimination of this chromatin from the new macronucleus.

It is clear that *Colpidium colpoda* differs but slightly from *C. campylum* as to its nuclear phenomena during binary fission. In the former the macronucleus and the central ball of chromatin are more regular and unvarying as to shape and staining capacity. There it is also possible to identify more definitely the central ball of the early stages with the bud of extrusion chromatin that is cast out after plasmotomy.

Glaucoma scintillans

The resting nuclei of *Glaucoma scintillans* are similar in shape and proportions to those of *Colpidium colpoda*. The chromatin of the micronucleus is more compact, however, and the macronuclear chromatin appears to be arranged in a rather loose reticulum (Fig. 6, *A*).

As the micronucleus swells in the prophase of mitosis a small, compact sphere of deeply-staining chromatin makes its appearance in the center of the macronucleus (Fig. 6, *B*). This ball enlarges to a considerable extent during the later prophase, metaphase, anaphase, and telophase of the micronucleus, retaining for a long period its strong affinity for nuclear dyes (Fig. 6, *C*, *D*, *E*, and *F*). As the two daughter micronuclei move apart the macronucleus begins to elongate in the direction of the long axis of the cell. At the same time the central ball of chromatin becomes less basophilic and elongates so that it lies across the center of the macronucleus (Fig. 6, *G* and *H*). The macronucleus constricts on either side of the central ball and the resulting configuration is very similar to that of the equivalent stage of *Urocentrum turbo* (Fig. 6, *I*). The two constrictions do not, however, proceed to completion. The central ball divides into two approximately equal halves (Fig. 6, *J*) and this division plane becomes the plane of fission dividing the macronucleus into two daughter halves (Fig. 6, *K*). The chromatin of the central ball retains its identity, as in the case of *Colpidium colpoda*, but unlike this species does not fragment until after plasmotomy. After the daughter ciliates separate, the chromatin of the central ball contracts and forms a distinct bud on the macronucleus (Fig. 6, *L*). This bud usually fragments into a number of spheres as it is being pinched off from the macronucleus (Fig. 6, *M* and *N*). As in *Colpidium campylum* and *C. colpoda*, the products of the extrusion bud round up, become homogeneous and deeply staining and are dispersed into the cytoplasm (Fig. 6, *O* and *P*). The disintegration spheres grow smaller and smaller until they are ultimately absorbed.

DISCUSSION

The papers dealing with the divisional activities of the four species of holotrichous ciliates used in this investigation are extremely scarce.

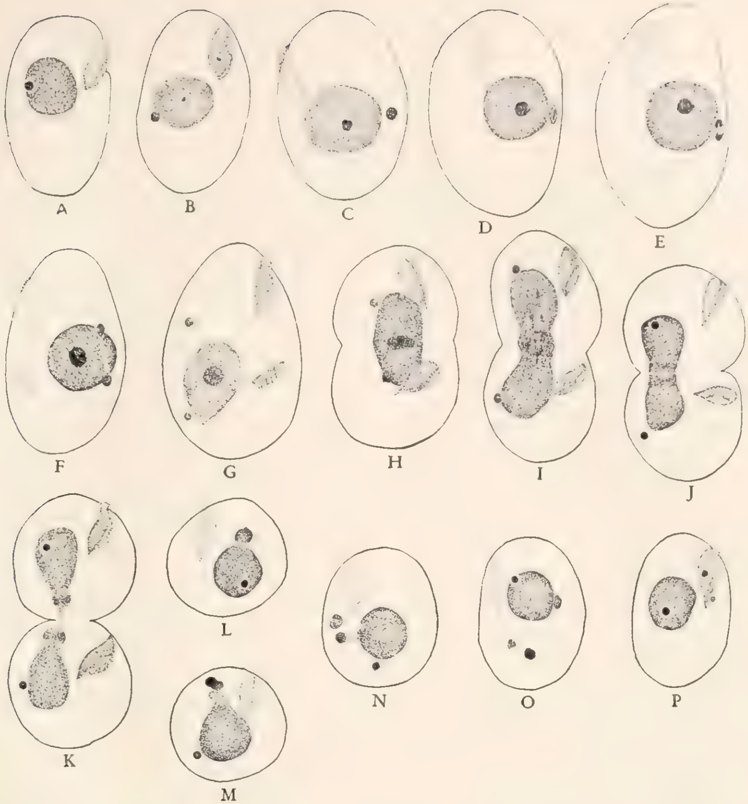

FIG. 6. *Glaucoma scintillans.* Camera lucida drawings from material fixed in Schaudinn's fluid and stained with the Feulgen nucleic acid reaction. × 567.

A. Resting individual.

B. Early division stage. The micronucleus is in the early prophase and a small bead of central chromatin has appeared in the macronucleus.

C. Slightly later than *B.*

D. Later stage. The central chromatin is large and deeply staining and the micronucleus is in the late prophase.

E. The micronucleus is in the anaphase and the central chromatin is at its maximum size and concentration.

F. Telophase of the micronucleus. The cytostome of the posterior daughter ciliate has made its appearance in its characteristic transverse position.

G. Later stage than *F.* The macronucleus is starting to elongate.

H. Further elongation of the macronucleus. The central chromatin mass is beginning to stretch across the center.

I. Constriction of the macronucleus. The central chromatin forms the swelling in the region of constriction.

J. Division of the central chromatin mass.

K. Further constriction and separation of the daughter macronuclei and the halves of the chromatin mass.

L. Daughter ciliate shortly after division. The central chromatin is budding off from the macronucleus.

M. Later stage of the budding and fragmentation of the central chromatin.

N. About the same stage as *M.*

O. Later stage in the reorganization of a daughter ciliate. The fragments of the central chromatin are degenerating in the cytoplasm.

P. The nuclei have reorganized. Two small fragments of the central chromatin are still in evidence.

That fact seems strange when we consider the wide distribution and ease of obtaining these common forms. The few studies that have been reported in the past have either completely missed the visible macronuclear reorganization involving the casting of chromatin into the cytoplasm or have failed to connect this phenomenon with normal binary fission. This failure is perhaps not strange when it is noted that the vast majority of accounts of macronuclear division include stages only up to the actual separation of the daughter ciliates. If the daughter macronuclei separate at a single division plane, it has been assumed that the daughter halves will take on the trophic form without further complications. This has been described repeatedly as the normal holotrich manner. As one may see from the above account of division of *Colpidium campylum*, *C. colpoda*, and *Glaucoma scintillans* the " normal manner " is followed regularly by a profound series of activities before the daughter macronuclei become reorganized.

We are aware of no work that has been reported on the cytology of the nuclei of *Urocentrum turbo,* although Haas (1933) mentions the occurrence of a " Zwischenkorper " during the division of *Loxocephalus* and *Urocentrum.* The appearance of the extrusion chromatin during the division of the macronucleus of *Loxocephalus* was given by Behrend (1916), but we can find no record of a like report for *Urocentrum* so we must conclude that the note by Haas refers to his own observations.

It is quite plain that the type of chromatin extrusion exhibited by *Urocentrum turbo* is the same in all respects as that shown so regularly in a number of holotrichous commensals. These forms have been previously summarized (Kidder, 1934) and include members of the Conchophthiriidæ and the Ancistrumidæ together with *Ichthyophthirius* (Haas, 1933).

Resch (1908) gave a very excellent description of the division of *Colpidium colpoda.* Unfortunately we have had access to the text only, as the figures were not included in the copy of his work that we were able to obtain. He described the formation of the central ball of deeply staining chromatin in the macronucleus of early division forms. This ball he referred to as the chromatin nucleolus or karyosome. He noted the elongation of the " karyosome " across the division nucleus but he followed the process only to the separation of the daughter ciliates. He therefore stated that the macronuclear division is clean. However, in the second part of his paper he described in some detail the actual extrusion of the central chromatin. He interpreted this as a degenerative process and referred to the ciliates showing macronuclear budding as " depression organisms." Because of their small size and the budding of the macronucleus, he believed that these ciliates were in a degenerate state and that the budding was the result of the upset of the karyoplasmic ratio. He interpreted the degenerating spheres of chromatin as micronuclei and reported the number as variable, up to six. It seems clear, however, that his " depression organisms " represent the normal daughter ciliates in post-divisional reorganization, and the variable numbers of micronuclei are in reality the degenerating spheres of macronuclear chromatin. (We have found as many as eight.)

The next report of division in *Colpidium* was that of Prowazek (1915). He devoted a single paragraph to the process and contributed little of interest.

The only other investigation of a cytological nature on the division of *Colpidium* is that of Ivanić (1928). He was mainly concerned with the details of the micronucleus and described spherical chromosomes. As mentioned earlier, we have failed entirely to confirm his findings. He treated the macronucleus only cursorily, describing it as dividing cleanly.

Very little has been written on the division of the genus *Glaucoma* as regards its cytological details. The only reference that we were able to find on *Glaucoma scintillans* pertaining to its division was that of Prowazek (1908). He noted the similarity between *Glaucoma* and *Colpidium* as to division but his report is very incomplete. He was especially interested in the division of the cytostome and the reorganization of that region in the daughter ciliates. He described the transverse appearance of the mouth in the posterior daughter before plasmotomy, an observation that we have been able to confirm (see Fig. 6). No details of the action of the nuclei were given.

Just how widespread among the holotrichous ciliates is this phenomenon of post-divisional chromatin elimination can only be determined by careful examination of many other types, particular attention being paid to the daughter ciliates immediately after plasmotomy. If the eliminated chromatin represents worn-out material, as was suggested by Kidder (1933*a*, 1933*b*, 1934), then the more profound the cleaning out of this material the nearer the cell will be to perfect reorganization. Such perfect reorganization may be at least a partial explanation for both the ease of culturing and the high division rate of *Colpidium* and *Glaucoma*. Also we would like to point out that in the three species that exhibit post-divisional chromatin elimination we have, as yet, observed no signs of conjugation. We know from previous accounts that conjugation does take place, at least in some strains, but these species certainly do not conjugate as freely or as often as the great majority of forms. It seems possible that the profound macronuclear reorganization that takes place at each division may, to a certain extent, restore the cell to its fundamental condition and decrease the necessity for conjugation.

It is quite feasible that post-divisional chromatin elimination may take place in *Boveria*. According to Stevens (1903), a central ball forms between the daughter macronuclei at the plane of fission. This ball divides and one-half fuses with each daughter macronucleus. Stevens did not observe the daughter ciliates after plasmotomy, so it is not known whether the fusion is permanent or whether the chromatin of the central ball later emerges.

Summary

1. *Urocentrum turbo, Colpidium campylum, Colpidium colpoda,* and *Glaucoma scintillans* all exhibit the phenomenon of macronuclear chromatin elimination during the process of binary fission.

2. *In Urocentrum* a ball of chromatin is differentiated from the elongating macronucleus. This ball contracts in the region of the plane of fission. As the daughter macronuclei separate, the ball is left between the two as a residuum. The residual ball is absorbed into the cytoplasm usually before the daughter ciliates separate.

3. *Colpidium campylum, C. colpoda,* and *Glaucoma scintillans* exhibit a peculiar type of chromatin elimination, practically identical in all three species.

4. A ball of deeply-staining chromatin forms in the center of the macronucleus during the prophase of the micronucleus. This ball increases in size, and comes to occupy the region of the division plane. Instead of remaining intact, as the macronucleus divides the ball also divides, one-half going to each daughter macronucleus. After the daughter ciliates separate, the chromatin of the central ball is budded off and cast into the cytoplasm, where it degenerates.

5. This type of post-divisional chromatin elimination is the first reported for holotrichous ciliates.

6. It is suggested that the profound reorganization of the macronuclei of *Colpidium* and *Glaucoma* may account for their high division rates and also for the infrequency of the appearance of conjugation.

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THE RELATION BETWEEN THE POSITION OF THE FEMALE PRONUCLEUS AND THE POLAR BODIES IN THE UNFERTILIZED EGG OF ARBACIA PUNCTULATA

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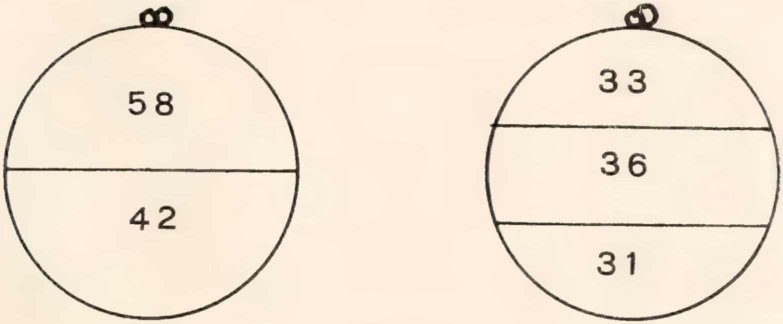
Investigators working with the egg of the sea urchin, *Arbacia,* have known for some time that the position of the female pronucleus cannot be depended upon in determining the polar axis of the egg. The location of this cell organ varies greatly in relation to that of the transient micropyle and to that of the polar bodies which occasionally are to be found attached to the surface of the egg. Insofar as I am aware, no record has as yet been made of the relative frequency with which the egg nucleus is found in one part of the cytoplasm as opposed to another. The jelly of the egg swells in sea water so that the micropyle is not easily located in eggs which have been in that medium for any length of time. Moreover, the polar bodies have generally been lost by the time the eggs are recovered from the female. During the summer of 1933, while examining eggs by means of the cardioid dark-field condenser, one batch was found which showed polar bodies still attached to nearly all of the eggs. Under these conditions the relation between the female pronucleus and the polar bodies was easily seen and recorded.

Three sets of records were made of the eggs which, when taken together, prove that the nucleus may be found with nearly equal frequency in any part of the egg cytoplasm. This statement must be qualified, however, for it was consistently noted that the pronucleus was not to be found in the exact center of the egg but was always between the center and the cortex. It should also be understood that the figures on distribution to be given below apply to a stage which must follow the formation of the second polocyte by but a short interval. It is conceivable, though not probable, that the pronuclei tend to assume a more constant location in relation to the polar region of the egg as this interval increases. It would seem more reasonable to expect their distribution to be even more diverse if that were possible.

The first count of the eggs was made to determine the number of times the pronucleus is found in the polar hemisphere of the egg as contrasted with the apolar hemisphere. The results are to be found in

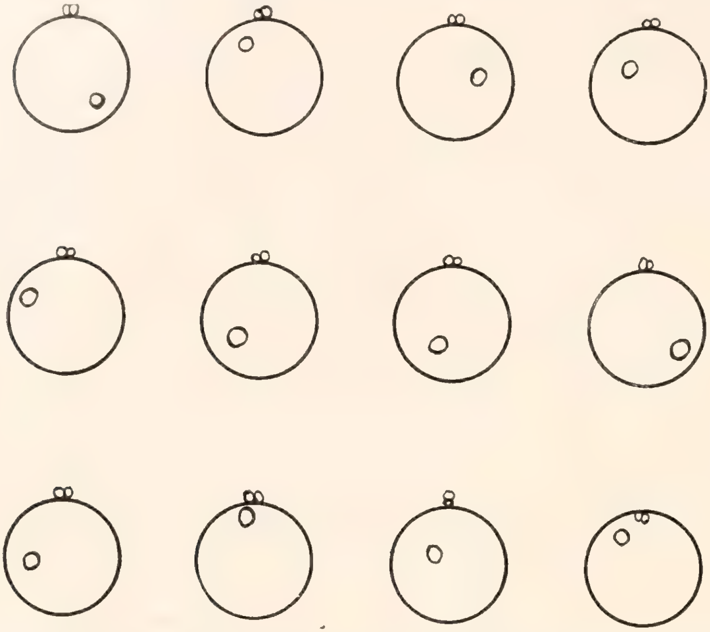
Diagram 1. From this diagram it might seem that there was a slight advantage of the polar over the apolar hemisphere, but the difference is so slight that its significance is at least questionable.

In making the second count, the egg was divided into three zones, one the third nearest the polar end, a second the middle equatorial band, and a third the lower apolar region. More of the egg cytoplasm is to be found in the middle equatorial third than in either of the polar thirds, which may well account for the fact that, as may be seen in Diagram 2, the pronucleus is more often found in the middle third of the egg than in either of the other zones. At the same time it is evident from the figures that the nucleus is found in the polar band more frequently than in the apolar. As in the case of the count for the two hemispheres, this difference may not be significant. On the other hand, the interval between the second meiotic division and the time of the observations may have been so short that the majority of the nuclei still remain slightly nearer the pole.



Diagrams 1 (left) and 2 (right). Showing the relative frequency in which the female pronucleus appears in different parts of the egg cytoplasm in relation to the position of the polar bodies; 100 eggs.

In addition to the counts made above, twelve eggs, chosen at random, were sketched to show the relations of polar bodies and nuclei. These are shown in text figure 1. It will be noted that of these twelve, seven show the nucleus in the polar hemisphere. When the egg is divided into the three zones it will be seen that the nuclei are equally distributed between the three. There is no indication in the sketches as to whether the pronuclei are located nearer the upper surface of the egg or the lower. At first, owing to the fact that they were consistently in the region between the center and the cortex, it was thought that some difference in density might have been influential in localizing them and that this orientation might have been lost when the eggs were transferred to the slide. Differences in density have been demonstrated for similar egg pronuclei in numerous experiments by E. B. Harvey and have been reported by her in a number of places. In order to test this, a mass of eggs was placed on a slide on the stage of the microscope and allowed to remain in place without being disturbed for one hour. At the end of that period there was no indication of any reorientation nor was there any constancy in position. ,A degree of force sufficient to influence the position of the nucleus apparently is attained only by the use of the centrifuge.



Text Fig. 1. Relative positions of the polar bodies and the female pronucleus in twelve eggs selected at random from those showing the polar bodies at the periphery of the egg as seen in surface view.

The results of these observations demonstrate that at that stage in the unfertilized ovum of *Arbacia punctulata* which follows closely the second meiotic division, the female pronucleus is located in the more superficial region of the endoplasmic cytoplasm and may be in any relation to the attached polar bodies. There is some indication that the nucleus is to be found nearer the polar end of the egg than the apolar end in the majority of the cases but the difference in the numbers of the eggs falling in each group is so small as to make this conclusion questionable.

REUNITION OF PIECES IN HYDRA, WITH SPECIAL REFERENCE TO THE RÔLE OF THE THREE LAYERS, AND TO THE FATE OF DIFFERENTIATED PARTS[1]

EMMA JOHNSTONE PAPENFUSS

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The work of H. V. Wilson and his associates (1907 and later) showed that dissociated cells in sponges and hydroids may reunite to form masses which may regenerate into complete normal individuals. The present paper is a study of related phenomena in the fresh-water hydra.

Reunition of small cut pieces of hydra was first studied by Issajew (1926). The present author published in 1932 an abstract giving certain results of the work here presented. Weimer (1934) has recently published a further study of this matter.

The purposes of the present work are somewhat different from those of Issajew and Weimer. Issajew was interested primarily in the forms of the individuals reconstituted from union of the mixed fragments of several individuals, and in the factors determining the forms taken. Weimer is interested mainly in the relation of the reconstitution processes to axial gradients, and in reformation of new polarities and axes. In making the present study, the main questions under consideration were the following:

1. May isolated cells thus reunite to form a complete individual, or must there be small masses of tissue containing representatives of the three layers of which hydra is composed?

2. Just what is the rôle of the three tissue-layers—ectoderm, mesoglea, and entoderm—in the process of fusion of parts in hydra?

3. What is the fate of differentiated tissue cells in the production of the regenerated individuals? This is a question which has been much studied in the work on sponges and hydroids. Three possibilities have been discussed: Is the regenerated organism produced exclusively from undifferentiated cells present in the original individual—all the differentiated cells being destroyed during regeneration? Or do the diverse types of tissue cells persist, reappearing as cells of the same type in the

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[1] This work was aided by a grant from the Brooks Fund of The Johns Hopkins University.

regenerate? If this is the case, do they retain their typical features throughout the process of regeneration, or do they become temporarily de-differentiated, each later resuming its typical features? Or finally do the diverse types of cells become de-differentiated into some generalized type, from which the differentiated cells of the regenerate are later produced anew, irrespective of the source of the generalized cells?

The fresh-water hydra possesses marked advantages for the study of these questions, owing to its simple structure. It possesses but two layers of cells, the ectoderm and entoderm, united by the non-cellular mesoglea. The undifferentiated cells are limited to the so-called interstitial cells, located between the bases of the ectodermal cells. The structure is thus favorable for determining the rôle of the different types of cells.

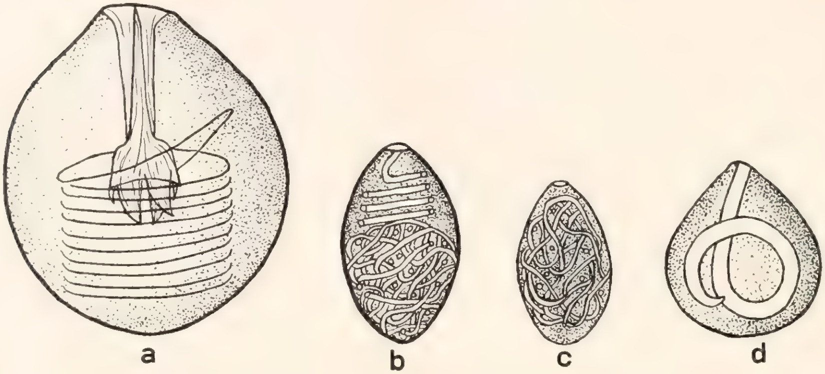

FIG. 1. Drawings of the four types of nematocysts found on the brown hydra employed in the experiments: *a*, penetrant; *b*, streptoline glutinant; *c*, stereoline glutinant; *d*, volvent.

Both the green and the brown hydra were employed in the present work. The green hydra is the common species, *Chlorohydra viridissima.* The brown hydra used does not agree with any of the species described in the monographic paper of Schulze (1917) nor with any of the species described in the taxonomic papers of Hyman (1929, 1930, 1931*a*, 1931*b*). Since Schulze uses the nematocysts as the basis of classification, it will be well to give a brief description, with figures, of the four types of nematocysts of the brown hydra here employed.

Penetrant (Fig. 1, *a*): 18–20 micra long, 15 micra wide. Cnidocil, 5–6 micra long. Apex of capsule slightly convex and characteristically slanted. The coil is easily distorted and hence it is difficult to determine its characteristic shape. In some specimens it is concave, in some convex, while in others it does not appear to be curved. Three large spines, one superimposed, and two short spines are evident. When the capsule is lying so that the three large spines can be seen it is characteristic to see a diagonal thread, which is a continuation of the coiled thread, across the capsule. The diagonal thread bends on itself and descends, apparently connecting on the other side of the capsule. The exact connection of the thread could not be determined.

Streptoline glutinant (Fig. 1, *b*): 12 micra long, 6 micra wide. Cnidocil, 10 micra long, very slender. Capsule oval in shape. Thread makes 3 or 4 transverse, usually slightly oblique coils. Below these coils the thread is wound in a tangled mass.

Stereoline glutinant (Fig. 1, *c*): 10 micra long, 5 micra wide. Thread wound irregularly.

Volvent (Fig. 1, *d*): 10 micra long, 8 micra wide. Cnidocil, 10 micra long. Characteristic to have thread halfway cross itself again as shown in Fig. 1, *d*. Capsule pyriform.

In the present paper this form will be designated simply as "the brown hydra."

Attempts to Obtain Regenerates from Expressed Tissue

The method of obtaining dissociated cells introduced by Wilson for sponges and hydroids consists in enclosing pieces of tissue in bolting cloth and forcing the cells through the interstices. This method is difficult to apply to hydra, owing to its small size. A method used by Dr. Florence Peebles for hydroids was more applicable to hydra. This consists in forcing the tissues through a small cloth sieve. The method was employed as follows: Bolting cloth was placed over the end of a small glass tube 7½ mm. in diameter. This tube was fitted into an outer tube 9 mm. in diameter. The hydras were expressed through the cloth by the use of a glass rod rounded at one end. For each experiment 30 to 40 hydras were used.

The perforations of the bolting cloth used by Wilson measured about 300 micra. When cloth with such perforations was employed for hydra (examples with perforations of 400 micra and of about 265 micra were tried), large pieces of hydras, whole tentacles, and entire buds came through. Therefore cloth of a finer mesh was used. Silk cloth with the perforations measuring about 40 micra gave a much finer precipitate. Under the microscope the precipitate was found to consist of free entodermal cells, free ectodermal cells, and groups of ectodermal or entodermal cells. Free algal bodies and nematocysts showed that many of the cells had been broken. The amount of entodermal tissue that comes through is much greater than that of ectodermal tissue, possibly because the ectodermal cells adhere to one another more firmly and hence do not pass readily through the perforations.

Such expressed tissue was cultured in spring water in a Stender dish, half filled with a firm agar jelly. The tissue was brought together in a small hole in the agar, produced by pressing into it the rounded end of a warmed glass rod; in this way it was insured that the bits of tissue would be in contact.

However in no case were observable fusion masses formed. The cells did not unite and no regeneration of individuals occurred. It appears probable that the failure to form fusion masses was due to special conditions resulting from dissociation of the cells. It was noticeable that the small pieces found in the expressed tissue contained groups of either ectodermal or entodermal cells, but rarely contained both kinds of cells attached together. It appears possible that in order to form fusion masses there must be pieces that had both ectodermal and entodermal cells held together by the mesogleal layer, in the usual way. Neither Issajew (1926) nor Weimer (1934) produced individuals from tissue thus expressed through fine perforations, although Issajew (p. 44) alludes to a report that an unnamed Russian student had succeeded in doing this. In order to determine whether pieces carrying all three layers can by union form a normal hydra, the experiments set forth in the following sections were performed. (It may be noted that at this time the author was unfamiliar with the work of Issajew, and Weimer's paper had not appeared.)

Reunition of Small Cut Fragments to Form an Individual

In these experiments the hydras were cut with sharpened needles into many small fragments. Such fragments contain ectoderm and entoderm, united by the mesogleal layer, as usual. In practice, for these experiments, the anterior end, with its mouth, hypostome, and tentacles, was removed, so that the fragments consisted only of parts of the body and foot. The fragments were transferred with a fine pipette to an agar bed made in one of the cavities of a depression slide. Only a little water was transferred with the fragments, so that they lie close together in relations favorable to fusion. As the fragments are somewhat scattered when expelled from the pipette, they are brought closer together with a fine pointed glass needle. In some cases the fragments were carefully oriented with entodermal faces upward, so that the three layers would be in the same relations in all the fragments. In other cases the fragments were left as they came from the pipette, with varying orientation of the different layers. The number of fragments was as a rule about 25 to 30 but, in some cases, the hydra was cut into 60 fragments or more. The slide bearing the fragments in close contact was placed in a moist chamber and observed at intervals. A small drop of spring water was gently added every few minutes.

In such slides containing small cut fragments, fusion of the fragments occurred, and eventually entire hydras were regenerated from them. Complete fusion commonly occurred in one to two hours. These results were obtained in many experiments with both the green and the brown hydra. The exact processes in fusion and regeneration are taken up in later pages.

According to Peebles (1897), the smallest portion of a hydra that is capable of independent regeneration is a sphere whose diameter is $\frac{1}{6}$ mm. It was not practicable to measure each fragment in all the experiments, but in a few cases in which the fragments were measured and all kept under this minimum size the usual regenerate was formed. Thus it is possible for an individual to form from fused fragments which alone are not capable of regeneration.

Here we are confronted by the question: Why do fusion and regeneration occur among small fragments that are cut apart, while with fragments obtained by pressing hydras through fine-meshed cloth they do not occur? As we saw earlier, in the cut fragments the three body layers retain their usual relations, while in the expressed tissue they do not. The difference in the results in the two cases calls for a study of the rôle played by the three body layers and their relations to each other, in fusion of tissues in hydra. Such a study is presented in the next section.

Rôle of Entoderm, Mesoglea, and Ectoderm in Fusion and Grafting in Hydra

In order to determine the rôle of the three body layers in fusion of parts, the process was studied by other methods than those employed in following the regeneration of new individuals from separate fragments. At first grafts were made, and an attempt made to follow in fixed and stained sections the rôle of the different layers. Grafts involving two green hydras, and in other cases grafts between a green and a brown hydra, were thus studied, under fixation with osmic acid and staining in safranine A and fast green. But such sections revealed nothing of the processes by which the respective layers became united in the grafts.

Success was reached by methods which separated in the living condition the three layers, and allowed the capacity for fusion in each to be tested with precision. The method employed was based partly on the ancient discovery by Trembley (1744) that hydras can be turned inside out, so that the entoderm is on the exterior. By placing in contact two such everted hydras, it becomes possible to determine whether two entodermal layers in contact will fuse. The mesogleal layer may be tested in a similar way by scraping the entoderm from two everted hydras and bringing them into contact. By placing these free mesogleal and entodermal layers in contact in different cases with other free layers, six combinations can be tested: (1) entoderm with entoderm; (2) entoderm with ectoderm; (3) entoderm with mesoglea; (4) mesoglea with mesoglea; (5) mesoglea with ectoderm; (6) ectoderm with ectoderm. The results for each combination are given in later paragraphs.

For obtaining the free surfaces of entoderm and mesoglea, the following procedure was employed. To evert a hydra, it was placed head downward in a hole made in an agar bed in the hollow of a depression slide. The foot was then pushed downward through the mouth with a glass needle until the hydra was everted. If the free surface of the mesogleal layer was desired, the everted hydra was taken from the hollow and allowed to extend. Then the entoderm was carefully removed with a very fine glass needle. In this way a body wall was obtained consisting only of the ectoderm and mesoglea. Sections of hydras so treated show the mesoglea adhering to the ectoderm, and without entoderm.

For the fusion experiments such specimens were transferred to another agar bed, in contact with another specimen. Close contact was insured by withdrawing most of the water. The slide bearing the specimens was placed in a moist chamber and observed at intervals during fusion; a little water being added every few minutes to prevent drying.

Entoderm with Entoderm

When two everted hydras are placed side by side with entoderms in contact, fusion occurs immediately. No irritation of the tissues is required and the fusion occurs more quickly than in ordinary grafting. In great numbers of such experiments fusion has in no case failed to occur. Fusion of the two entodermal layers is so complete that in sections there is no line of demarcation between the two original layers. Fusion may thus occur between the entoderms of the green and brown hydras, but such fusion occurs less readily than that between members of the same species.

Entoderm with Ectoderm

In these experiments a specimen was everted and brought, with its entoderm external, in contact with a hydra that was not everted. Entoderm and ectoderm were thus in contact. The specimens were left in contact 20 to 70 minutes. In no case did fusion of entoderm and ectoderm occur, though such experiments were tried in great number.

Entoderm with Mesoglea

When an everted "entodermal hydra" is placed in contact with a "mesogleal hydra" (mesoglea on its outer surface), union always occurs. The affinity between these layers (which of course are normally closely united) is striking. There seems to be a direct adherence, as compared with the comparatively slow union of entoderms, which is a process occurring in several stages. (In my abstract of 1932 it was erroneously stated that entoderm and mesoglea do not unite).

Mesoglea with Mesoglea

In a large number of experiments the mesogleal layers were brought in contact, in two everted hydras from which the entoderm had been removed. In no case was there a firm or lasting adherence. Sometimes there was a slight tendency to hold together at definite points or over small areas. This usually occurred about the mouth, a region from which it is difficult to completely remove the entodermal cells. It is clear that no functional adherence such as will lead to a lasting union occurs between two mesogleal layers.

Mesoglea with Ectoderm

A hydra is everted, the entoderm removed, and the exposed mesoglea brought in contact with the uninjured ectoderm of another individual, remaining thus for 20 to 70 minutes. In no case does fusion occur.

Ectoderm with Ectoderm

When two hydras are placed with the respective ectodermal surfaces in contact, fusion does not occur, even though they are kept in close contact 30 to 60 minutes.

Thus entoderm fuses readily with entoderm and with mesoglea, but not with ectoderm. Mesoglea does not fuse with mesoglea nor with ectoderm. Ectoderm does not fuse with ectoderm nor with either of the other two layers.

The Process of Fusion of the Entodermal Cells

The experiments just described have shown that the entoderm is the layer which is mainly active in fusion (the uniting of entoderm to mesoglea can obviously play little part in the union of fragments into a reunition mass). The process of union of the entodermal cells was studied in detail in tissue cultures.

For this purpose a hydra was everted, several fragments of entoderm cut off with a fine glass needle, and these again into a number of smaller pieces. These pieces were placed close together on a cover-glass in a small drop of water, and the cover-glass was inverted over a depression slide. Water was allowed to run in under the cover-slide in order to seal it. In this way the processes occurring in fusion could be observed under the microscope.

Fusion occurred so rapidly that it was not possible to make camera drawings or photographs from the living cultures. Therefore cultures were killed and fixed at different stages; for this purpose methyl acetic green was found useful. It stops the process of union instantly and fixes the tissues beautifully, in the condition they have reached.

The striking characteristic of small fragments of entoderm is their amoeboid movement. Clear hyaline processes are sent out from each fragment and shortly afterward the granular protoplasm with its contained green algal bodies flows out into them. The fragments at times also undergo a revolving movement; this happens both in large and small fragments (ten to thirty micra in diameter). This movement is like that resulting from cilia or flagella: it may be due to the cilia known to occur on the entodermal cells (Schneider, 1890, and others).

When fragments of entoderm come in contact they usually fuse rapidly, though rarely they separate again without fusion. The cells are apparently not brought together by an attraction between them, since fragments that are very close together may wander apart again. They apparently come in contact only accidentally as a result of their irregular movements.

Though the process of fusion is readily observed, it is at first difficult to determine just how it occurs. Camera drawings of different stages of the process were made, and by the study of these light was thrown on the method. When two fragments come in contact, a process from one piece slides over a process from the other. Other processes are sent out, and the same thing happens, so that the processes become interlaced. In time they are so woven together that it would be difficult for them to be disentangled. In this way the fragments are brought into union. Figure 2 shows two entodermal fragments in the process of fusing. After a few minutes fusion is complete and the two fragments are united into one. The cellular membranes of the two fragments do not break and allow the interior protoplasm of the cells to mingle; on the contrary, the cells retain their integrity and are united at first only by their interlaced processes, possibly supplemented later by a physical adherence of the cell surfaces.

The relations just brought out appear to explain the fact that fusion and regeneration did not occur in hydra tissue expressed through cloth.

while it did occur in the case of small fragments cut with a sharpened needle. The expressed material is composed largely of entodermal tissue that has become separated from the ectodermal tissue. Fusion thus occurs only between adjacent masses of isolated entodermal tissue; this was observed in the cultures made. But such masses, composed exclusively of entoderm, are not capable of regenerating an entire hydra; they soon die. Even though there might be present in the tissue expressed through cloth some fragments containing the three layers in their normal relations, their fusion would usually be prevented by the intervening masses of ectodermal cells, and dying or dead entodermal cells. Hence it would be difficult to obtain a fusion mass capable of regenerating a normal hydra from tissues dissociated by forcing them through fine cloth; in none of the experiments here tried did this occur.

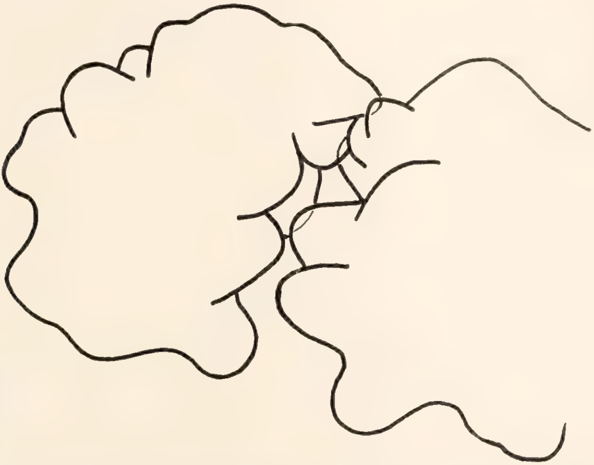

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FIG. 2. Camera lucida drawing showing two fragments of entoderm in the process of fusing. From a fixed preparation stained with methyl acetic green. × 300.

Examination of the accounts of wound healing and of grafting in the work of other investigators shows them to be consistent with this conclusion that it is the entoderm that plays the active rôle in fusion of parts. Thus in the work of Ischikawa (1890), it was found that when an everted hydra is placed within a normal hydra and the two are pierced with bristles a permanent union occurs; but when an uneverted hydra is placed within another hydra and the two pierced with bristles, union is obtained only where the bristles pierce the two hydras: that is, where the entoderms of transplant and host can come in contact (pp. 445–446, and Figs. 61–65, Taf. 19). It has often been noted that in order to obtain union between two hydras, it is necessary to "irritate" the tissue:

that is, to wound the surface; such wounding doubtless exposes the entoderms, which then unite. Rand (1900) observes that if the surface of but one of the individuals is wounded, union does not occur; also that if after wounding the surfaces of both, they are allowed to heal and so become covered with ectoderm before being brought in contact, union does not occur. He remarks that " In most of the experiments the two pieces were at first united by the adhesion of fragments of entoderm " (p. 203). The work of Mattes (1925*b*) on wound healing in hydra shows similar relations. In the healing of a wound " usually the entodermal layers first come in contact and unite " (p. 33). Then the ectodermal cells gradually grow or creep over the entoderm till they come in contact. In Mattes' Fig. 8 is shown a section in which the entodermal edges of the wound have united, but are not yet covered by ectoderm.

It appears probable that the ectoderm plays, in the union of fragments to form a hydra, a rôle similar to that in wound healing, as described by Mattes. The ectoderm has no capacity for active fusion, such as is shown by the entoderm. However, after the entodermal parts have fused, the ectoderm in regenerating grows over these entodermal surfaces.

Summary on the Rôle of the Different Tissues in Reunition

Our evidence up to this point makes it possible to answer a number of the questions proposed at the beginning:

(1) In hydra, isolated cells do not reunite to form a complete individual. For such reunion and regeneration there must be small masses of tissues containing representatives of the three layers of which hydra is composed. (2) In fusion the active rôle is played by the entodermal cells, which unite by sending out processes which interweave, the ectoderm being later regenerated from the preëxisting ectoderm and thus gradually covering the entodermal masses. (3) The diversity of entodermal and ectodermal cells persists through the process of fusion and regeneration. Whether there may be in addition some new production of either or both types from the interstitial cells, remains uncertain.

There remain certain questions as to the processes by which the heap of fragments unites and gradually transforms into a hydra, and as to the fate of differentiated structures in this process.

The Processes of Union, Regeneration, and Regulation by which the Fragments Form an Individual

To obtain successful fusion and regeneration, the following methods were employed. After the head had been removed, the body was cut into fragments by the use of two finely ground No. 12 sewing needles.

The fragments were then transferred to the agar bed on depression slides, with a pipette having an opening about 1 mm. in diameter, so as to convey little water and leave the fragments close together (see the photograph, Fig. 3). For successful fusion it was best to orient the fragments all with the same surface (ectoderm or entoderm) upward, and to fit them as closely together as possible with a needle—increasing or decreasing the small amount of water as needed. Fusion will often occur successfully even when the fragments lie at random, not all having the same surface upward: but results are more certain when they have like orientation. The slides are kept in a moist chamber until fusion occurs. This usually takes from one half to two hours. In early stages they must be examined every few minutes to prevent drying. In some of the fragments, by a curving of the edges, ectodermal cells come to lie at the region of contact with other fragments; in consequence fusion will not occur. In such cases the ectodermal edge may be trimmed away or pushed back into place so as to leave a free edge. To keep irregular fragments in good contact, small pieces of agar may be placed around them. Where there are gaps between the fragments, areas which fail to fuse may occur; these presumably give rise to the abnormalities later described.

These methods are successful with both the green and the brown hydra. The brown hydra was used for most of the experiments. In a number of cases attempts were made to obtain regenerates from a mixture of fragments from the two species, but in all such cases the fragments degenerated.

The further process will be described by the aid of a series of typical figures, shown in the photographic plate (Plate I, Figs. 4 to 8).

Fusion begins among the fragments lying on the agar bed (Fig. 3); after about two hours they have formed a flat plate, one side being ectoderm, the other entoderm (Fig. 4). Usually there are a number of regions in which fusion is not complete.

When the plate stage is reached, the tissue is taken from the agar bed and placed in a Stender dish filled with spring water. As soon as it is free in the water, there occurs a movement by which the edges are rolled inward toward the entodermal surface as shown in Fig. 5. (This is best seen if placed with entoderm above). In this way in a half hour a half-open hemispherical structure is produced. Sections at about this stage or a little later show that the apparent gap in the cylinder is covered with entodermal cells, over which there may be a thin layer of ectoderm. The resemblance is close to the sections figured by Mattes (1925) in his study of wound healing in hydra. Mattes' studies show clearly that the opening is first closed by entoderm, which later is covered by regenerating ectoderm growing over it from the sides.

Changes continue, and about three hours after the formation of the plate, the hydra is a flattened sac-like body, hollow within (Fig. 6). An indication of a point at one end shows the position of the foot.

Three days later the foot is serving as an organ of attachment, and one tentacle has appeared. This and slightly later stages are shown in Figs. 7 and 8. The tentacle elongates and the hypostome appears. Other tentacles are developed. As will be set forth later, there are frequently irregularities and abnormalities at this time, probably the result of incomplete fusion.

At this stage the hydras begin to feed. They were fed once a day the soft parts dissected from a daphnia. If they are fed regularly and the water is changed every one or two days, they now live indefinitely, regulate their abnormalities, and reproduce by budding. The processes by which the hydra finally reaches its normal form may best be taken up in connection with an account of the fate of differentiated structures. It may be noted that green hydras usually give more complete fusion and fewer later abnormalities than the brown hydra.

Regulation and the Fate of Differentiated Structures, in the Formation of a New Hydra from Fragments of an Old Individual

What becomes of the differentiated parts that are present in the individual cut into fragments? Do the fragments from a given part of the body form anew that part? Or are the differentiations lost and formed anew, without relation to the source from which the fragments come?

A beginning on the study of this question was made by tracing the

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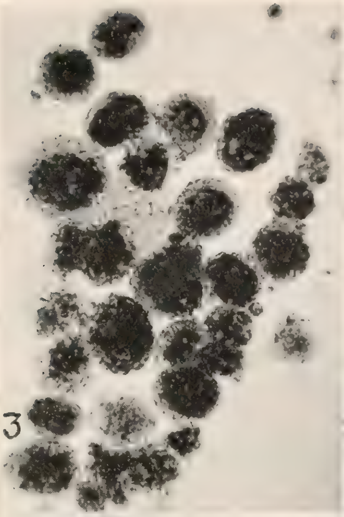
EXPLANATION OF PLATE I

Photomicrographs of representative stages in the formation of a green hydra from fragments. Figures 5 and 9 are photomicrographs from another experiment in which a brown hydra was used.

3. Green hydra fragments, prior to fusion. × 80.
4. Well-advanced fusion of fragments. × 200.
5. Reunition mass (formed from brown hydra fragments), after it has been placed in water, showing the rolling-in of the edges. × 320.
6. Later appearance of reunition mass after structure has become sac-like. × 200.
7. The appearance of the regenerate after three days. The foot is attached. Note the developing tentacle at the broad end. × 80.
8. The appearance of the 4-day regenerate. The hypostome has formed and the tentacle has elongated. × 80.
9. A brown hydra regenerate which has been formed from fragments, with two attached feet. × 26.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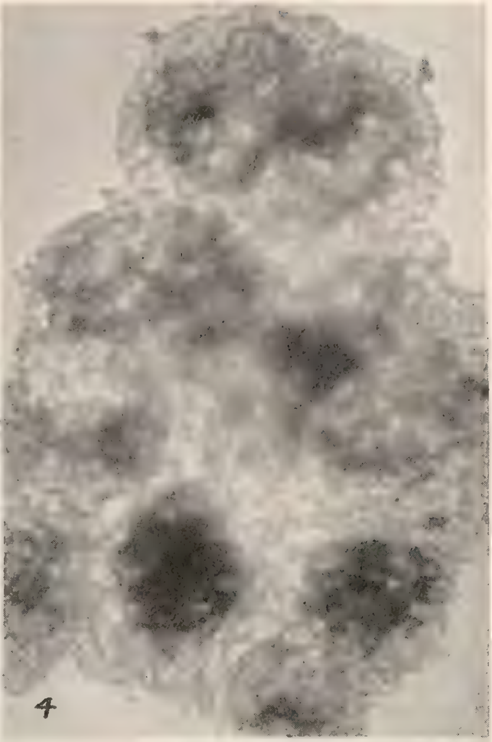
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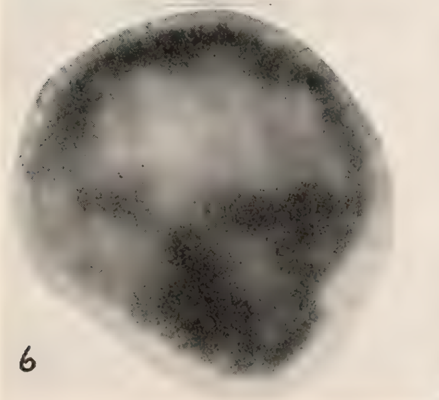
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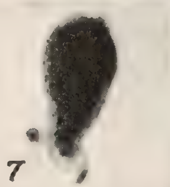
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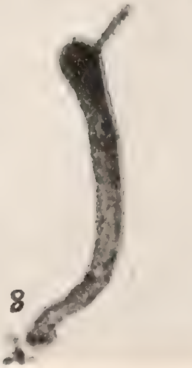
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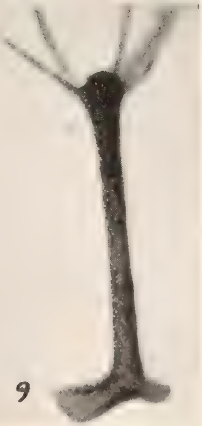
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PLATE I

fate of the original foot. This study throws much light on the nature of the regulations that occur.

An attempt was first made to follow the fate of foot fragments of the green hydra when the rest of the mass came from the brown hydra; and in the reverse combination. In such cases the green foot unites with the brown fragments, and is still present in the plate stage. Thereupon, however, the green foot is invariably sloughed off, and the same thing happens in the reverse combination.

Recourse was then had to the method of vital staining. For this the brown hydra is best, since here the stained tissue is more readily seen. The foot is removed as a small fragment, and is stained in a deep solution of methylene blue. The remainder of the body is cut into fragments without staining. The foot fragment is repeatedly washed, then placed with the other fragments in the usual way. It enters into union with them, and its fate may be followed, since it is stained blue.

It should be remarked that in following the development, the blue stain gradually fades. After six or seven days the faintly blue foot area may be restained. The regenerating mass is placed in the agar bed, a minute crystal of methylene blue is taken on the end of a fine steel needle and placed exactly on the stained area, where it is allowed to remain for a few seconds. The blue color is thus restored. The regenerating mass is then plunged into spring water to remove excess stain. The original foot area may thus be restained repeatedly.

All the experiments in which the staining allowed the foot to be traced throughout showed that the original foot retains its identity through the fragmentation, fusion, and regeneration. It is not absorbed nor transformed into some other tissue. It becomes the foot of the regenerated individual, functioning in the usual way. In some cases the foot retained its adhesive function throughout the entire process; in young spherical masses the stained foot could be seen to adhere to the substratum.

Sometimes an additional foot, or more than one, was formed from tissue which did not come from the original foot. This shows, of course, that a change of function may occur in other cells of the body. But such a change did not occur in the foot tissue in any of the numerous experiments in which the stained material could be followed from beginning to end.

The presence of a stained area representing a differentiated portion of the original hydra aids greatly in tracing the regulative processes by which a new individual is produced from the heap of fragments. Probably in consequence of failure of complete fusion, abnormalities are commonly present in the young regenerate. These are less common in the green hydra: here frequently only a single hydranth (mouth and hypostome with the circle of tentacles) and but one foot are produced. But in the brown hydra usually two or three hydranths and one or more accessory feet are present in the early stages. The regulative processes affecting the foot are bound up with those in other parts of the body.

In some cases the original foot forms at the beginning the basal end of the long axis. In this case it preserves its position through the often complex regulative processes that occur, and becomes the foot of the regenerated hydra. Thus, in Experiment 16, made upon a brown hydra, the original foot *F* was from the first at one end of the long axis (Plate II, Fig. 10). Four hydranths, *A*, *B*, *C*, *D* appeared, all upborne by the foot, but none of them opposite it (Fig. 11). One of the hydranths migrated till it was opposite the original foot (Fig. 12), and the other three coalesced. A splitting then occurred through the middle of the one hydranth which had been formed by coalescence (Fig. 13), separating the hydra into two parts, *X* and *Y*. As a result the original foot functioned as the permanent foot of the hydranth, *X*, which was closest to it (Fig. 14). The other part formed a new foot (*AF*, Fig. 13).

In another case (Experiment 22), in which the original foot *F* was from the beginning at the basal end of the long axis (Fig. 15), three hydranths developed: two, *B* and *C*, opposite the original foot, and one, *A*, at right angles to it (Fig. 16). Two accessory feet also developed from material that did not belong to the original foot, one AF_1, opposite the anterior hydranth and adjacent to the original foot, the other, AF_{11}, at the anterior end of the long axis (Fig. 16). Later coalescence set in between the two anterior hydranths *B* and *C*, and also between the two posterior feet, one of which was the original foot. The third hydranth *A* migrated toward the anterior end and coalesced with *B* and *C*. The accessory foot, AF_{11}, migrated downward and united with the foot that had formed by the coalescence of the accessory foot AF_1 and the original foot *F*, so that the final foot was formed by the union of three originally separate feet, two of which were newly formed (see the conditions shown in Figs. 17 and 18).

In many cases the original foot is not at first at one end of the long axis, but located somewhere on the body wall. In such cases appropriate regulatory changes occur, bringing the foot to the basal end of the axis; these may be of varied character. In some cases regulation is by migration of the foot. Thus, in Experiment 25, the original foot formed an appendage in the posterior part of the body wall (Fig. 19) while a new foot *AF* was formed at the opposite end from the regenerating hydranth. A bud, *b*, later appeared posterior to the original foot, near the new foot,

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EXPLANATION OF PLATE II

Figures 10–14. Camera lucida drawings of representative stages in the formation of regenerate 12.

10. One-day regenerate. *F*, original foot; *N*, non-fusion area. × 20.

11. Three-day regenerate. *F*, original foot; *N*, non-fusion area; *A, B, C, D,* developing hydranths. × 22.

12. Fifteen-day regenerate. Hydranths *A* and *B* are coalescing. *F*, original foot. × 22.

13. Twenty-seven-day regenerate. Hydranths *A, B,* and *D* have coalesced. A split has occurred, separating into two parts the hydranth formed by the coalescence of *A, B,* and *D*. An accessory foot *AF* has developed. *X, Y,* polyps formed by the splitting of *A, B,* and *D*. × 10.

14. Thirty-day regenerated individual, *X*, with the original foot serving as a permanent foot. *F*, original foot. × 10.

Figures 15–18. Camera lucida drawings of representative stages in the formation of regenerate 22.

15. One-day regenerate. *F*, original foot. × 22.

16. Five-day regenerate. Three hydranths *A, B,* and *C* and two accessory feet AF_1 and AF_{11} have developed. *F*, original foot. × 22.

17. Nine-day regenerate. The original foot *F* and the accessory foot AF_1 are beginning a coalescence. The accessory foot AF_{11} is migrating toward the posterior end of the regenerate. The hydranth *A* is migrating toward the anterior end. *F*, original foot. × 22.

18. Twenty-nine-day regenerate. The accessory foot AF_1 has coalesced with the original foot *F*. And foot AF_{11} is beginning a coalescence with AF_1 plus *F*. On the fortieth day AF_{11} coalesced with AF_1 plus *F* so that the newly-formed foot was composed of the original foot *F* and the accessory feet AF_1 plus AF_{11}; *b*, developing bud. × 10.

Figures 19–22. Camera lucida drawings of representative stages in the formation of regenerate 25.

19. Six-day regenerate. The original foot *F* is an appendage-like structure on the polyp. An accessory foot *AF* has been formed. × 22.

20. Eight-day regenerate. A bud *b* has developed posterior to the original foot *F*. × 10.

21. Eleven-day regenerate. The bud *b* has constricted off. The original foot *F* is migrating toward the basal end. × 10.

22. Twenty-day regenerate. The original foot *F* has coalesced with the accessory foot *AF*. The newly-formed foot of the polyp is composed of the accessory foot *AF* and the original foot *F*. × 10.

Figures 23–30. Camera lucida drawings of representative stages in the formation of regenerate 17.

23. Three-day regenerate. Two hydranths, *A* and *B*, have developed. The tentacles of *A* are not drawn. *F*, original foot; *N*, non-fusion area. × 10.

24. Six-day regenerate. The foot is no longer an appendage-like structure but has become incorporated. *F*, original foot; *A, B,* hydranths; *b*, developing bud. × 10.

25. Ten-day regenerate. The short axis has now become the long axis and the foot is toward one end of the long axis. The regenerate is not yet attached. Hydranths *A* and *B* are beginning a coalescence. The bud *b* has constricted off. *F*, original foot. × 10.

26. Twenty-five-day regenerate. The two heads, *A* and *B*, have practically coalesced. A constriction is appearing just posterior to the original foot. An accessory foot *AF* has developed. *F*, original foot. × 10.

27. The posterior end of the 26-day regenerate. The constriction posterior to the original foot is increasing. *F*, original foot. *X*, part that is constricting off. × 22.

28. The posterior end of the 28-day regenerate. *X* constricted off and lived for several days. *F*, original foot. × 22.

29. The posterior end of the 31-day regenerate. The extreme posterior end which is not original foot tissue is gradually rounding off. *F*, original foot. × 22.

30. The re-formed foot of the 33-day regenerate. The original foot tissue forms part of the new foot. *F*, original foot. × 22.

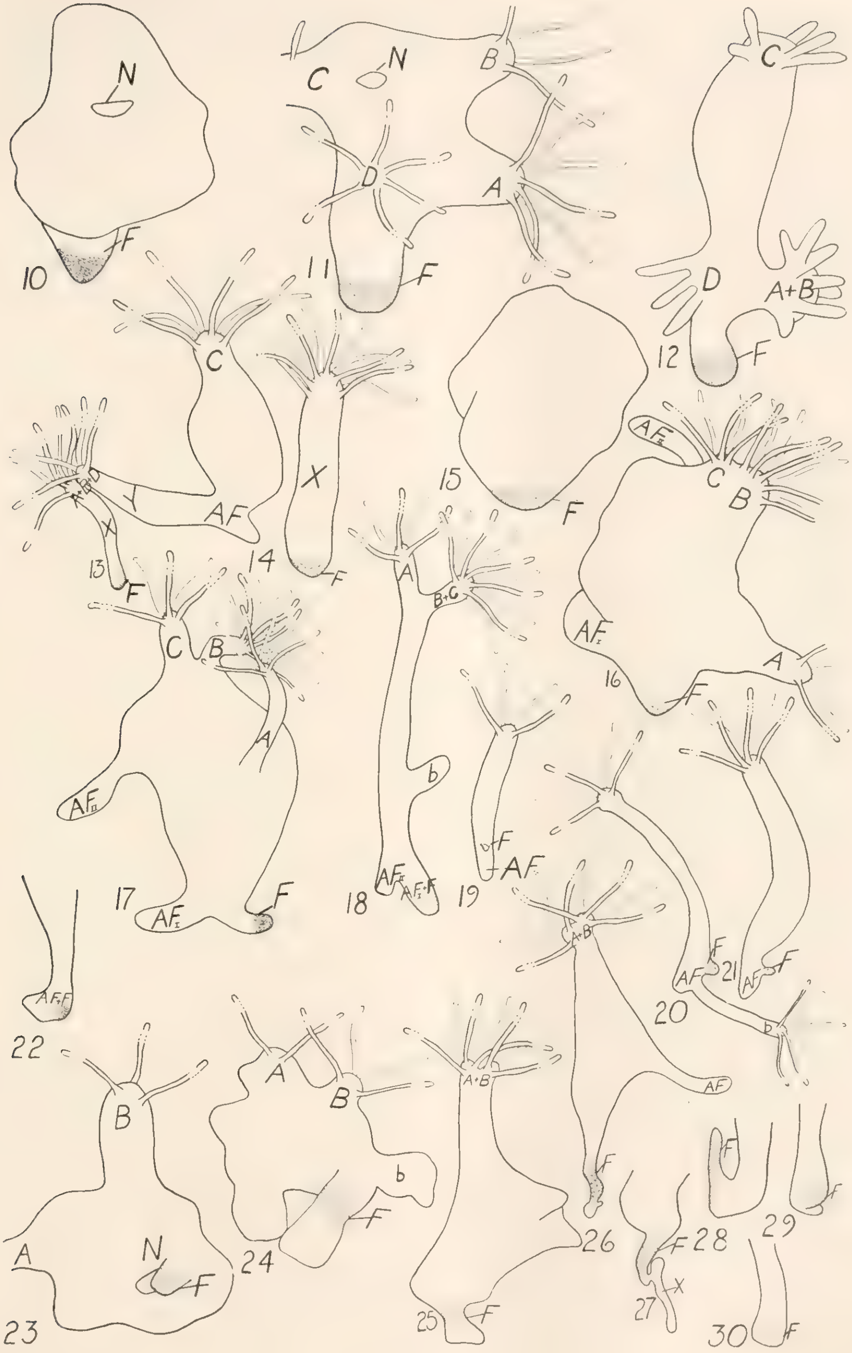
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PLATE II

in a region much below the usual budding zone (Fig. 20). After this bud had constricted off, the original foot migrated downward and finally coalesced with the new foot (Figs. 21, 22).

In one remarkable case the original foot *F* was attached on the short axis of the very irregular reunition mass, and two hydranths, *A* and *B*, appeared opposite the foot, thus likewise on the short axis (Fig. 23). The foot, at first an appendage, became incorporated in the body wall (Fig. 24). After 8 days the body in this region became narrow: the entire region anterior to the foot elongated, and the two heads approached one another, so that the regenerate showed more resemblance to a normal hydra, with the foot toward the posterior end of what was now the long axis (Fig. 25). Thus the axis had changed, and was now at right angles to the original longitudinal axis; this seems to have occurred under the influence of the differentiated foot. The foot was, however, still not at the end of the long axis, a part of the former body wall region forming the basal end. Sometime later a constriction appeared posterior to the foot (Fig. 26), and in a few days the part of the body behind the foot was constricted off, leaving the original foot in the normal position at the basal end of the long axis (Figs. 27, 28, 29, 30).

In attaining the final normal form a very great variety of regulatory processes occur. The types of regulatory processes in hydra have been fully described by Rand (1899, 1900), Issajew (1926), Tripp (1928), and others, so that it is not necessary to present details here. By way of summary, the following may be said. The regulatory processes may be classified in three types: separation or splitting of parts; absorption of parts, and migration with coalescence of parts. Splitting or separation of parts is relatively infrequent; two examples have been given in the descriptions just presented. Absorption and separation of parts has been found of common occurrence by other investigators (e.g. Rand, 1899; Tripp, 1928), but it was rare in these experiments.

Migration and coalescence of parts was by far the most frequent method of regulation. These two processes always occurred together; before coalescence can occur there must be migration of certain parts. Separate hydranths coalesced, separate feet coalesced, separate tentacles coalesced. Careful study revealed that this was the usual method of getting rid of supernumerary parts: it occurred in practically all the experiments. Coalescence occurred only between like parts. Parts at a distance from each other (two feet or two hydranths) migrate together as if they were attracted one to the other, and finally coalesce. When the number of tentacles is doubled by the coalescence of two heads, the number is reduced to normal by the further coalescence of the tentacles.

SUMMARY

1. When tissues of hydra are expressed through bolting cloth having meshes as large as those used for sponges and hydroids, large masses of tissue, entire tentacles, entire buds and the like, come through so that this method is not adequate for hydra.

2. When hydras are expressed through cloth of fine mesh (40 micra), detached and fragmented cells, and masses in which entoderm is separated from ectoderm, are produced. Such cells or fragments do not reunite to form a new individual.

3. However, small cut fragments (25 to 60 from the body of an individual) under favorable conditions reunite to form a new hydra. Such fragments carry both entoderm and ectoderm united in the usual way.

4. To determine the rôle of the body layers in the reunition of parts, methods were devised for exposing each of the three layers separately and bringing them each in contact with one another. This gave the following results: Entoderm unites readily with entoderm and also with mesoglea, but does not unite with ectoderm. Mesoglea adheres occasionally to mesoglea, but does not unite with ectoderm. Ectoderm does not fuse with either of the other layers, nor with another layer of ectoderm.

5. In the union of fragments to form a new individual, the initiative is taken by the entodermal cells, which send out protoplasmic processes which interweave with one another. The gaps in the ectodermal layer are seemingly covered by regenerative growth of ectoderm, spreading over the united entodermal masses. This explains why reunition does not occur when entoderm and ectoderm are separated into different fragments.

6. Thus the diversity of ectoderm and entoderm persists through the process of fusion and regeneration. Whether additional production of either or both types from the interstitial cells occurs was not determined.

7. A reunition mass formed by uniting fragments of the green and brown hydra disintegrates. Or if a small amount of tissue from one species is united with a large amount from the other, fusion occurs, but the kind of tissue that is present in small amount later sloughs off.

8. The fragments first unite to form a plate with ectoderm on one side, entoderm on the other. The edges then roll together to form a cylinder, with entoderm within. Head and foot are then produced.

9. To determine the fate of differentiated parts, the foot of the original hydra was stained with methylene blue, so that the fate of the fragment which it constitutes could be followed. The original foot retains its identity and function, later becoming the foot of the individual re-

generated from the fragments. But accessory feet may also be produced from other tissues.

10. Many irregularities and abnormalities are produced in the early stages of regeneration, probably as a result of incomplete fusion in some regions. Supernumerary heads, feet, and tentacles are common, and form and structure are frequently abnormal.

11. These abnormalities are later regulated by: splitting of parts, absorption of parts, and migration and coalescence of parts. The two former methods are unusual, as most regulation involves migration and coalescence. Only like organs coalesce.

ACKNOWLEDGMENTS

This work was carried out under the direction of Professor H. S. Jennings to whom the writer is greatly indebted for aid and criticism. She is further indebted to Professor R. P. Cowles and Professor E. A. Andrews for many helpful suggestions while the experimental work was in progress.

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THE CHEMICAL NATURE OF THE AMPHIBIAN ORGANIZER

I. The Use of the Cephalin Fraction of Mammalian Brain as an Inducing Agent

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Introduction

A number of recent papers have thrown some light upon the chemical nature of the substances which induce a neural plate in the amphibian embryo. The work of Holtfreter (1933) has shown that the dead organizer functions after treatment with xylol or 100 per cent alcohol but loses its ability to induce a secondary neural plate in *Triton* after treatment with ether. Further, Spemann, Fischer and Wehmeier (1933) showed that the organizer is not destroyed by acetone. Finally Needham, Waddington, and Needham (1934) were able to obtain induction in *Triton* with an ethereal extract of neurulæ. These researches indicate that some substance or substances soluble in ether but probably insoluble in alcohol, xylol, or acetone are responsible for the early embryonic induction of the neural plate.

Since the chemistry of amphibian neurulæ is not well known, it was thought that extracts of the mammalian brain, a structure which Holtfreter (1933) has shown to have powers of induction, would give more information about the chemical nature of the organizer. The chemistry of the mammalian brain has been extensively studied, and the cephalins have been analysed by Levene and Rolf (1922) and Page and Bülow (1931).

Ether extraction of the brain gives chiefly sterols, lecithins, and cephalins. The sterols are soluble in acetone, which leaves the lecithin and cephalin. The lecithin may be separated from the cephalin since it is soluble in alcohol, which precipitates cephalin. Bearing in mind that the organizer is an ether-soluble substance and probably insoluble in alcohol and acetone, we might expect that the cephalin fraction of the ether-soluble substances would act as an organizer. This was shown to be the case by the following procedure.

METHODS

One-half of a freshly killed calf's brain was chopped fine and extracted for twenty-four hours at 5° C. with 500 cc. of ether. The suspension was centrifuged and the clear ether extract evaporated to about 25 cc. at room temperature. Then 250 cc. of 100 per cent alcohol were added with the formation of a precipitate, and the flask was placed at 5° C. for 24 hours. The precipitate was separated from the solution containing lecithin by centrifuging and was redissolved in ether. Not all of the precipitate dissolved, and the residue after 24 hours in ether at 5° C. was discarded. The cephalin was reprecipitated from the ether by addition of acetone to remove cholesterol. The precipitate, consisting chiefly of cephalin, was redissolved in ether and once more precipitated with 100 per cent alcohol to remove lecithin and cholesterol. The precipitate was again dissolved in ether and reprecipitated with acetone to remove cholesterol. Finally the precipitate was redissolved in ether and allowed to evaporate to dryness at room temperature. The substance obtained was a white flaky compound which readily formed an emulsion with water. The cephalin obtained is obviously impure and further experiments are planned with better preparations. In the experiments described here, the cephalin was used 30–40 days after preparation, and during this time must have taken up oxygen.

The work was started in 1933 with *Ambystoma opacum,* which lays its eggs in September and October; but the animals stopped laying before extracts could be tried, so the experiments were continued with the black Mexican axolotl (*Ambystoma mexicanum*). *Ambystoma opacum* is an excellent form for induction experiments as it is very easy to operate upon in the early gastrula stage. There is very little mortality (see Table I). On the other hand, the Mexican axolotl is much more difficult to work with, as the early gastrula tends to flatten out over the wax or celophane-covered bottom of the dish, and the mortality is higher. However, by operating in Holtfreter's solution, about 50 per cent of the early gastrulæ survived through the formation of the neural plate.

Preliminary experiments on these forms showed that the living dorsal lip and the dead dorsal lip induced neural plates (Table I and Plate I, Fig. 4, I). In *Ambystoma opacum* eight clear cases of induction were obtained with the transplant of the living dorsal lip and in axolotl two cases are recorded.

The dead dorsal lip was prepared by desiccating early gastrula and implanting small pieces of the dorsal lip area into the blastocœl of a living gastrula. In all 23 implants of dried dorsal lip were made, resulting in 5 clear cases of induction and one doubtful case.

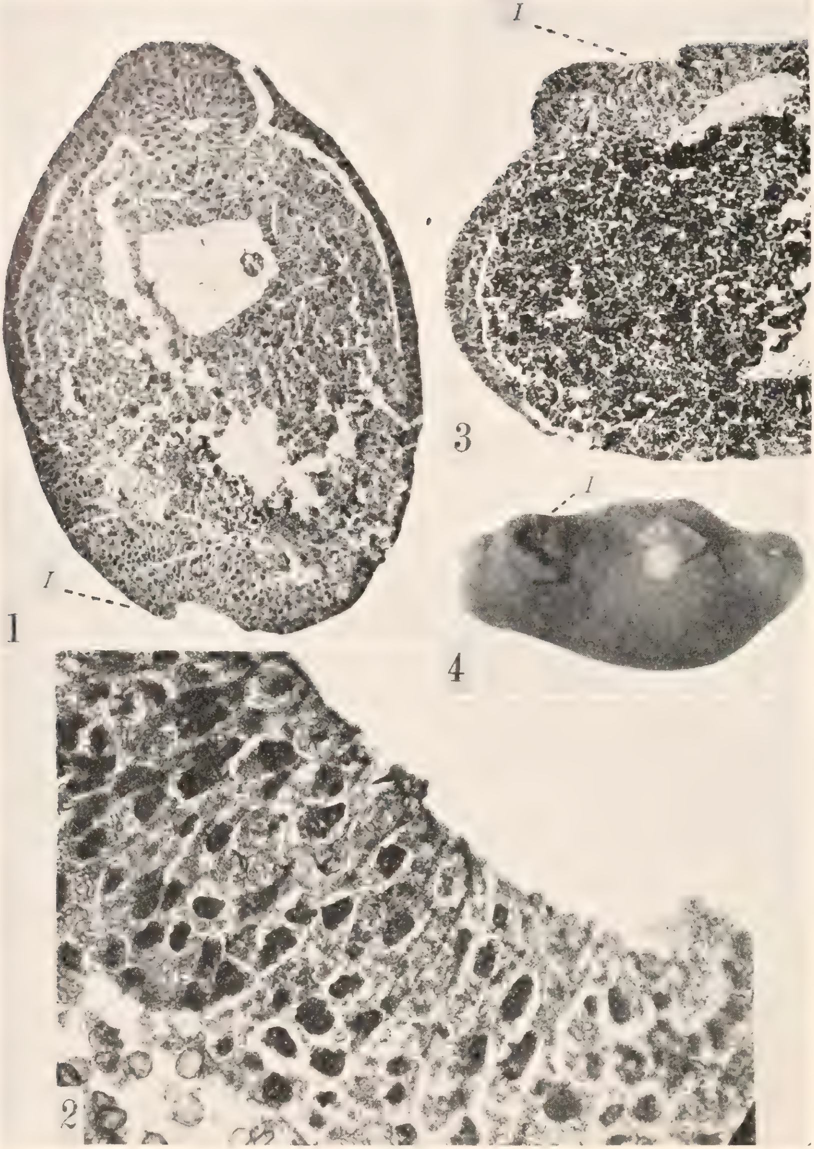

PLATE I

FIG. 1. Cross-section showing induced neural plate (I) in *Ambystoma mexicanum* (Mexican axolotl) by means of cephalin. Fragments of the implant may be seen under the induced neural plate.

FIG. 2. A detail of the induced neural plate of Fig. 1, showing the typical arrangement of cells and elongate nuclei.

FIG. 3. Induced neural plate (I) in *Ambystoma mexicanum,* using an ether extract of mammalian brain. Note especially the implant of the ether extract + kaolin just beneath the induced neural plate.

FIG. 4. Induced neural plate (I) in *Ambystoma opacum* with the living dorsal lip of the blastopore.

In all cases the implants were placed in the blastocœl of an early gastrula, according to the well-known technique in *Triton*.[1] Since the substances used formed emulsions with water, it was difficult to transfer them to the blastocœl under water. For this reason the extracts in most cases were mixed with an equal amount of kaolin to form a paste in water which, when dried, could be cut into small pieces and these pieces inserted into the blastocœl by means of sharp forceps. The extracts used were the cephalin preparation and also the entire ethereal extract which was evaporated to dryness.

RESULTS

Table I summarizes the results and Plate I, Figs. 1–4, shows photographs of the inductions. Figure 1 shows the primary neural tube and an induced neural plate in axolotl. The scattered fragments of the implant of cephalin and kaolin can be seen underneath the induced neural plate. In Fig. 2 the typical arrangement of the cells and the elongated nuclei of the induced neural plate are shown. Most of the eight clearly defined inductions obtained by cephalin were of this character. The four cases labelled as doubtful were ones in which a thickening of the ectoderm was obtained, but no typical neural plate resulted.

TABLE I

Induction of a neural plate in *Ambystoma opacum* and in the Mexican axolotl (*Ambystoma tigrinum* or *mexicanum*). Clear induction = Figs. 1 and 3 in Plate I. Doubtful induction = large masses of cells which could not be distinguished from epidermis.

Form used	Implant	No. operated	No. survived	Clear induction	Doubtful induction
Ambystoma opacum............	Living dorsal lip	13	13	8	1
Mexican axolotl................	" " "	4	2	2	0
" "	Dead (dried) dorsal lip	23	11	5	1
" "	Entire ether extract + kaolin	10	4	2	0
" "	Cephalin + kaolin	37	22	8	4

No controls were made with kaolin, since in two of the eight cases induction was obtained with cephalin alone. Furthermore, in *Triton* much evidence has been obtained that inert substances, such as agar, wax, gelatine, etc., have no inducing powers.

Only two cases of induction with the entire ether extract of mammalian brain are recorded here. Figure 3 shows a very clear induced neural plate (I) with the implant just below it. The implant consisted of 50 per cent ether extract and 50 per cent kaolin, which formed a solid mass that could be placed in the blastocœl. The ethereal extract was a portion of that from which cephalin was isolated. It was merely allowed to evaporate to a gummy, reddish mass and was then mixed with kaolin.

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[1] I am deeply grateful to Professor O. Mangold for demonstrating his methods of operating on *Triton*.

DISCUSSION

The experiments show that cephalin, or some impurity in it, will induce a neural plate in axolotl. It is possible that lecithin or cholesterol is present along with the cephalin, and these substances have not been tested on axolotl. However, Needham, Waddington, and Needham (1934) have implanted cholesterol in *Triton* and obtained no signs of induction.[2] In regard to lecithin, Spemann, Fischer, and Wehmeier (1933) have implanted the yolk of the hen's egg with negative results. These results, of course, do not conclusively rule out the action of specific brain sterols or lecithins.

According to Page and Bülow (1931), cerebrosides and sphingomyelin may be present in cephalin if the alcohol is not carefully removed from the cephalin precipitate. Since these substances are described as insoluble in ether, they could be present in only small amounts. However, the possibility should not be discarded that these compounds may be the organizer, especially since Fischer and Wehmeier (1933) reported induction of neural plates with glycogen. Here, according to Needham, Waddington, and Needham (1934) and Holtfreter (1933), the induction was probably due to an impurity in the glycogen. This is evidence that the organizer can function in very low concentrations.

Considering cephalin itself, it is found to possess many of the properties of the amphibian organizers as indicated in the following summary:

Property	Cephalin	Organizer
Occurrence	Brain and most of tissues of mammals	Brain and most of tissues of mammals
Solubility in		
Ether	Soluble	Soluble
Alcohol	Insoluble	Insoluble
Acetone	Insoluble	Insoluble
Xylol	Soluble	Insoluble
HCl	Insoluble	Insoluble
H_2O	Fine emulsion which diffuses	Probably diffuses

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[2] In a more recent communication, Waddington, C. W., J. Needham, W. W. Nowinski, D. M. Needham and R. Lemberg (1934) found that the unsaponifiable fraction of crude ether extracts from adult newts or calves' liver would induce. The active principle could be precipitated with digitonin. (See *Nature,* vol. 134, p. 103.)

The properties of the organizer are obtained from the papers of Bautzmann, Holtfreter, Spemann, and Mangold (1932), Holtfreter (1933), Spemann, Fischer, and Wehmeier (1933) and Needham, Waddington, and Needham (1934). It should be pointed out that " insolubility " in any solvent means merely that the organizer will induce a neural plate after it has been treated with the solvent. This does not necessarily mean that the organizer is insoluble in the solvent used, but may mean that the organizer is in combination with some substance within the cell.

On the whole it is not possible at present to distinguish between cephalin and possible impurities in it as causing the induction in axolotl. I merely wish to point out here that cephalin possesses many of the properties of the amphibian organizer.

There are a few analyses of the phospholipins of amphibian eggs, and the work is summarized by Needham (1931). Plimmer and Kaya (1909) found that the lipoidal phosphorus in the developing egg of *Rana temporaria* declined from 26.2 per cent of the total phosphorus content to 20.2 per cent. Fauré-Fremiet and Dragoui (1923) reported a phospholipin content of 5.98 per cent dry weight of the entire egg of the frog. Parnas and Krasinska (1921) found that 23 per cent of the phospholipins was lost between fertilization and hatching.

Unfortunately the nature of the phospholipins has not been determined. However, since cephalin has a wide distribution and even appears in some plant phospholipins (Levene and Rolf, 1925), it probably is present in the axolotl egg. It will be extremely interesting if it turns out that a compound (a cephalin), which is found in large amounts in the adult nervous system, acts as an inducing agent forming the embryonic nervous system.

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THE GROWTH-PROMOTING ACTION OF CRUSTACEAN EYE-STALK EXTRACT

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From the eye-stalks of such crustaceans as *Palæmonetes, Callinectes, Cancer,* etc., there can be obtained by water extraction a substance (or mixture of substances), which, distributed by the blood stream, will lead to contraction of the chromatophores of crustaceans (Perkins, 1928; Koller, 1928), contraction of the chromatophores of fishes (Koller and Meyer, 1930; Kropp and Perkins, 1933*b*), and expansion of the melanophores of tadpoles (Perkins and Kropp, 1932). These and other reactions are suggestive of the action of pituitary secretions. But despite its pituitrin-like properties in its action on chromatophores, the crustacean eye-stalk extract does not act in any detectable way on the gonads of either male or female rats (Kropp, 1932).

From human pregnancy urine a substance, "auxin," is extractable which, with proper technique, produces reactions in decapitated coleoptiles of oats, and roots of *Lupinus,* identical to the ones obtained with "growth-substance" extracted from vegetal tissues (Kögl and Haagen-Smit, 1931; Navez, 1933). In some of its properties it is very similar to the "Rhizopin" described by Nielsen (1930).

The so-called "growth-substance" (Cholodny, 1924; Went, 1928) extractable from the tips of coleoptiles of *Avena, Zea,* etc., or from the root-tips of *Lupinus* (Navez, 1933), produces characteristic, non-specific effects upon the elongation of decapitated coleoptiles or roots (also Keeble, Nelson, and Snow, 1931) identical to the one brought about by "auxin."

The fact that eye-stalk extract has pituitrin-like effects and that the same pituitrin reactions can be obtained by means of extracts of pregnancy urine led us to investigate the possible action of eye-stalk extract on elongation of plant tissues. Also, as a preliminary step in the process of separation of the different components of a water extract of eye-stalks, it was decided to test its action on the elongation of coleoptile and root tissues with the usual procedure used in studies of growth-substance. If a definite, positive result could be obtained, it might provide a basis for assaying such extracts. It is evident that a method of assay is necessary if we are to decide such important problems as the conditions of formation and liberation of the eye-stalk hormone; and whether the substance is formed under the action of light or merely released from the eye-stalk on illumination. In addition, it would be of primary importance to follow quantitatively the changes in activity of this substance in solution and to study the possible action of light upon the substance itself.

The technique usually followed in studies of growth-substance, involving the use of agar blocks " loaded " with the substance under study, is not free of objections. It was thought preferable, nevertheless, to try out such a method, rather than to place the decapitated roots directly in the solutions of known concentration. However, in order to make this assay directly comparable with experiments on growth-substance, preliminary trials showed the possibility of obtaining a response by placing decapitated roots in solutions of known " concentration " of eye-stalk hormone as expressed in terms of number of eyes per ml. of extract (*cf.* Kropp and Perkins, 1933*a*).

In comparing the mode of action of eye-stalk extract and growth-substance, two contrasting responses of plant tissues are available: (1) The rate of elongation of the decapitated coleoptile of *Avena* is markedly increased by placing on the decapitated surface a tiny block of agar imbibed with growth-substance. (2) The rate of elongation of the root of *Lupinus* whose tip has been removed and replaced by a block of growth-substance-imbibed agar, is decreased. Both effects are markedly affected by either concentration or total amount of the substance made available to the tissues.

Two hundred eye-stalks of *Palæmonetes vulgaris* which had been thoroughly light-adapted were macerated in 1 ml. of distilled water, with the addition of a small amount of NaCl, boiled, centrifuged, and the clear or nearly clear extract decanted; there is usually slight turbidity due to incomplete salting out. The extract was then diluted to 4 ml. A volume of 0.02 ml. of the extract thus corresponded to one eye-stalk. The solution was sterilized. Volumes of 0.01 ml. were tested by injection in *Palæmonetes* adapted to a black background and each sample produced the characteristic maximal contraction of chromatophores. The sterilized vials, rubber-capped, were kept in the refrigerator at 2° to 3° C. until needed.

The action of this extract upon the growth of coleoptiles of *Avena,* and upon the elongation of the roots of *Lupinus,* was tested by a general technique used in many other experiments in this Laboratory (Navez and Robinson, 1932). Small cubic blocks of agar, 0.8 to 0.9 mm. on edge, were cut from a sheet of agar 8 mm. square, which had been in contact with 0.05 ml. of extract for 3 to 4 hours in a moist chamber at 2.5° C. The cutting is done with the aid of a double razor blade device, previously wiped through a block of paraffin. Coleoptiles of *Avena*, of an average length of 30 to 35 mm., were decapitated by means of a small knife with a sharp V-shaped notch (also paraffined). Portions of tips 1.5 to 2 mm. long were removed. The root tips of *Lupinus* were removed to a similar length. The coleoptiles used were of seeds grown in sawdust in small glass vessels. The coleoptiles passed through a glass collar fixed in a sheet of hard rubber which was attached to the glass vessel; this avoids accidental bending or displacement of the coleoptiles during manipulation. Measurement of the coleoptiles is carried on while they are in a glass trough lined with filter paper; one side of the glass trough is hinged, which permits removal of water of guttation. The roots, of an average length of 25 to 30 mm. when used, were grown in vials 100 × 25 mm., lined with moist filter paper except for a vertical "window"; the seeds were held in paraffined paper funnels attached to the vials by paraffin blocks.

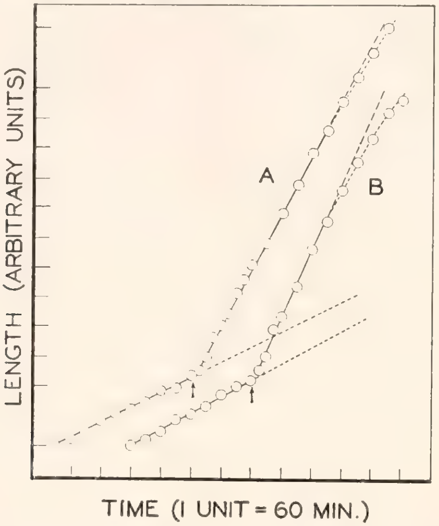

FIG. 1. Elongation of decapitated *Avena* coleoptiles under the influence of *Palæmonetes* eye-stalk extract. In this and subsequent figures arrow indicates point at which the extract-imbibed agar block was applied.

Decapitation and subsequent manipulation of the seedlings were carried out in dim red light. The agar blocks were placed at one side of the decapitated coleoptile, beside the projecting first leaf, with the help of a glass needle. The drop of water of guttation which appears on the uncovered half of the end of the coleoptile when the block is in place was removed at intervals by means of filter-paper strips; this prevented the displacement of the agar block.

For a preliminary trial young seedlings of *Lupinus* from which the root tip had been removed were " seated " on the smooth edge of small vials containing 2 ml. of extract. The decapitated root was immersed by about 5 mm. in the extract. Reactions were of the same order as with the agar blocks.

In Fig. 1 curves *A* and *B* show the elongation of two decapitated *Avena* coleoptiles which had been capped with blocks of agar imbibed with freshly prepared eye-stalk extract. The increase in rate becomes apparent within 15 minutes of the time the block is placed on the cut surface, and bears every similarity to the response of the decapitated coleoptile to the growth hormone itself.

It is important to consider that the extract from the eye-stalk contains NaCl and probably numerous other substances. Control observations were made with agar blocks imbibed with salt solution of the same concentration as that used in preparing the experimental extracts, and with extract of the abdominal musculature of light-adapted *Palæmonetes*. The extract of abdominal muscle was made by first washing in distilled water 100 abdomens, then macerating them without the addition of fluid. One milliliter of distilled water was then added to it with a few crystals of salt, the mass boiled and centrifuged, the supernatant liquid decanted and made up to 2 ml. Each 0.02 ml. of the extract thus corresponded to one abdomen. Injections of 0.02 ml. of the extract of abdominal muscle were made into both white-adapted and black-adapted *Palæmonetes,* and were found to be wholly without effect upon the chromatophores. It is apparent in Fig. 2, curves *A* and *B*, that the abdominal muscle extract has no action upon the elongation of the coleoptiles.

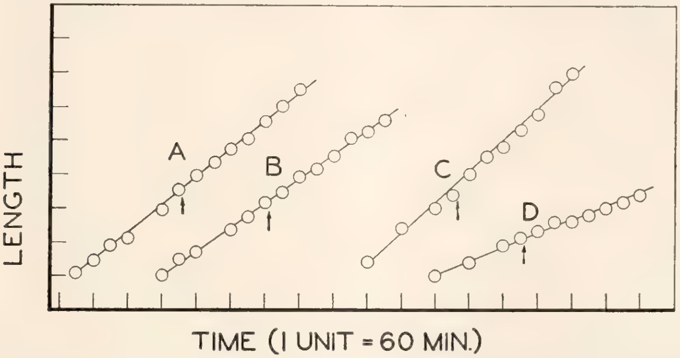

FIG. 2. Curves *A* and *B* show effect of *Palæmonetes* abdominal muscle extract upon elongation of *Avena* coleoptiles. Curves *C* and *D* show the effect of blocks of thoroughly leached agar without eye-stalk extract upon the elongation of *Avena* coleoptiles.

A definite reserve has, nevertheless, to be made here. In the experiments here reported all agar had been purified either by repeated distilled water extraction in the cold before gelation or by keeping aseptically the millimeter-thick layer of agar gel in contact with sterile distilled water, replaced every 24 or 48 hours for at least a week. As will be described by one of us in a paper in preparation, different batches of commercial agar either shredded, or U.S.P. powder, may yield different results under seemingly identical conditions. This might lead, of course, to strong doubt of the precision of the whole procedure, but it is possible, as shown by curves *C* and *D* in Fig. 2 (pure agar blocks), to prepare the agar gel in such a manner that no acceleration or deceleration effects are vitiating the reaction determined by the substance under study. Apparently commercial agars often contain traces of substances (salts, reducing substances, etc.) which sometimes have a slight effect on the elongation of the coleoptile. With some agars very low concentrations of NaCl will definitely exert a slight accelerating effect on the rate of elongation of the coleoptile, unless the agar samples are thoroughly repurified before gelation.

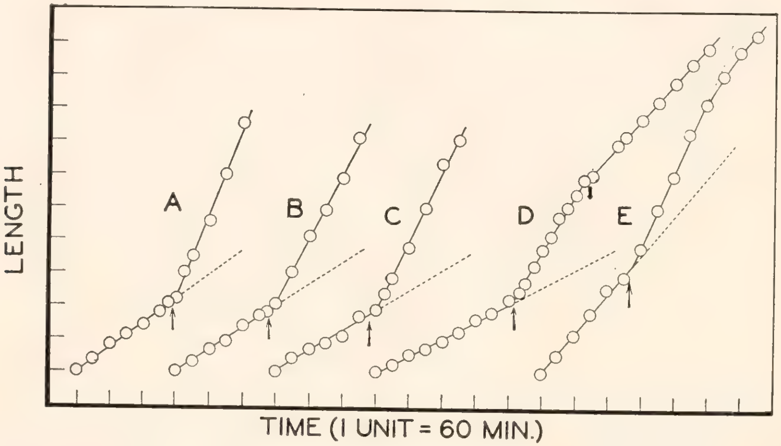

FIG. 3. Effect of aging of eye-stalk extract, not in contact with agar, upon rate of elongation of *Avena* coleoptiles. See text for details.

In order, therefore, to eliminate the possible accelerating effect of NaCl on coleoptile elongation, extracts were made from animals that had been washed free of sea water and the extracts made up without the use of NaCl. This gave a liquid which was usually slightly turbid although the chromatophore-activating and growth-accelerating qualities were not affected. Furthermore, all agar used was purified as described before. We conclude, therefore, that the acceleration effect produced by eye-stalk extracts prepared without the aid of NaCl and imbibed in thoroughly leached agar cannot be ascribed to NaCl nor to impurities in the agar.

All the experiments described previously were made with eye-stalk extract generally less than 12 hours old. On keeping vials containing aseptic extracts in the ice box at 2 to 3° C. for different lengths of time, one notes the deposit of a light gray flocculent precipitation. When kept at room temperature in the laboratory, this precipitate appears after 2 to 3 days, and is darker in color than the one obtained in the ice box.

That this precipitate is not of a proteinaceous nature is shown by the negative results obtained with Millon's reagent and with the xanthoproteic reaction. That it may be a denaturation product of some active substance is indicated by its very low solubility in water, even around 50° to 60°. Such aqueous extracts of the precipitate do not seem on evaporation to have dissolved any visible amounts of substance. The idea of a gradual breaking-down of the chromatophore activator of the eye-stalk extract is substantiated further by the following observations.

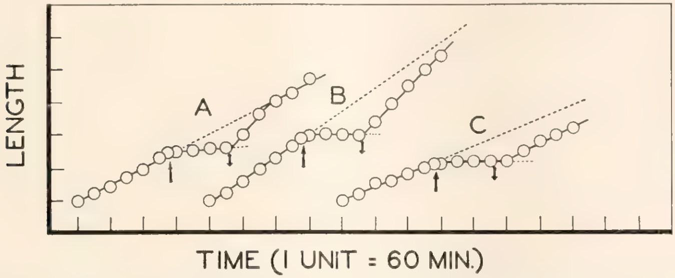

Fig. 4. Effect of aging of eye-stalk extract for various lengths of time in contact with agar blocks at 2° to 3° on the rate of elongation of coleoptiles with induced change in rate on removal of agar block. See text for details.

Figure 3 gives the results of a series of experiments all made under similar conditions, the only variable being the age of the extract, that is to say, the time elapsed between the completion of preparation of the extract and the moment when agar blocks are placed in contact with the solution. The time during which the sterile solutions are kept in the refrigerator increases gradually from 12 hours (Curve *A*), to 3 days (Curve *B*), 7 days (Curve *C*), 14 days (Curve *D*), and 25 days (Curve *E*). It is noticeable that up to about 14 days at least, there is very little difference in the reactions of coleoptiles. On the other hand, the activity of 25-days-old extract is definitely lowered. It may be noted in Curve *D* that the agar block was accidentally displaced with the consequence that a rate close to the rate prevalent before stimulation was resumed. As shown in Fig. 4, a totally different result is obtained if the solutions are in contact with the agar and kept in the refrigerator. Instead of an acceleration effect we get a deceleration, which is slightly more marked the longer the extract is in contact with the agar. Tentatively it may be assumed that in the presence of air and of some substance contained in the agar, the extract breaks down, yielding products strongly inhibitory.

These products must be of the same order of size as the accelerator product because the lag period one can observe from the moment of removal of the block is of the same duration as the one observed on application of the active block. Furthermore, the rate of formation of this inhibitor is indicated by the after effect exhibited upon removal of the agar block. For agar blocks in contact with the extract for 24 hours, Fig. 4 (Curve *A*), the initial rate is resumed after 1½ hours; agar and extract aged together 36 hours (Curve *B*) and 48 hours (Curve *C*) do not return to the initial rate within the duration of observation.

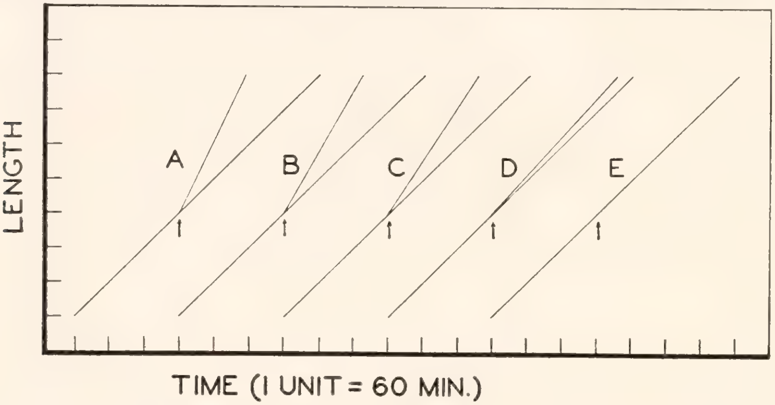

FIG. 5. Effect of concentration of eye-stalk extract upon elongation of coleoptiles. See text for details.

At present we are unable to say if light has an effect on the formation of this inhibitor, but we can point out that in the case of the experiments depicted in Fig. 1, where we find at the end of each curve a noticeable decrease of the accelerating effect, the whole process had been carried out under very dim red light during the periods of observation each lasting 3 to 4 minutes, followed by periods of total darkness (26 to 27 minutes). We are inclined on this basis to think that the gradual change in effect observed in long duration experiments is of the same nature as the one found by keeping imbibed agar blocks in the refrigerator. That the change in slope in Fig. 4 is to be ascribed directly to change in the concentration of the active material is demonstrated by the experiments shown in Fig. 5. Portions of a freshly prepared extract were diluted to such volumes as to have the series of concentrations 0.02 ml. (*A*), 0.04 ml. (*B*), 0.16 ml. (*C*), 0.64 ml. (*D*), and 1.28 ml. (*E*) per eye. The slope of each curve is markedly affected by the concentration of the solution under experiment.

It is of interest to note that the concentration of an extract of one eye-stalk in 1.28 ml. of fluid is well within the limits of crustacean chromatophore activation, although it shows no apparent coleoptile elongation effect. Despite the fact that the decapitated coleoptile tip of *Avena* is less sensitive to the eye-stalk hormone of *Palæmonetes* than the chromatophores of *Palæmonetes,* we have in the reaction of a plant to this hormone a precise and ready method of assay which has heretofore been lacking. Under the conditions described, the results are readily reproducible for any given lot of *Palæmonetes.* There remains, of course. the desirability of devising a method of assay which will also take into account the total mass of eye-stalk material used, since presumably the amount of active extract is dependent upon the mass of glandular or other tissue of the eye-stalk producing it.

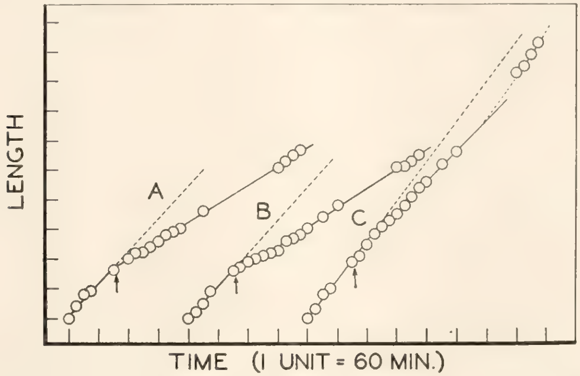

FIG. 6. Effect of eye-stalk extract upon elongation of roots of *Lupinus.*

Experiments to determine the effect of growth-accelerating material from the growing coleoptiles of *Avena* upon *Palæmonetes* chromatophores have thus far been wholly negative. The concentrations used represented 0.8 tip to 17 tips per milliliter of fluid extract, and no chromatophore effects were discernible with some thirty animals. Pregnancy urine was found to be without effect on *Palæmonetes* chromatophores. However, our tests were but preliminary and were carried out only with crudely refined material, injection of 0.3 ml. of which invariably caused death of the crustacean.

The action of eye-stalk extract upon the elongation of the root of *Lupinus* is illustrated in Fig. 6. The characteristic action of the plant growth hormone, at least in concentrations producing an effect upon the elongation of the *Avena* coleoptile such as we have described, is to reduce the rate of elongation of the root. This is also true of extracts of the root tip, when the agar block technique is employed. It is obvious in Fig. 6 that the effect of the eye-stalk substance is completely analogous in this respect. Evidence at hand indicates that younger roots (15 to 20 mm. long) exhibit more pronounced reactions than the older roots used for our experiments.

The authors acknowledge with pleasure the kind coöperation provided by Mr. O. E. Sette, in charge, North Atlantic Fishery Investigations, and the help of Mr. R. O. Goffin, Biological Collector, U. S. Bureau of Fisheries, in supplying us with living material.

SUMMARY

The chromatophore activator of the crustacean *Palæmonetes,* extractable from the eye-stalks, increases the rate of elongation of decapitated coleoptiles of *Avena.* The analogy of this effect with that due to the growth hormone normal to the plant is extended by the fact that the eye-stalk substance, like the substance of coleoptile tip or root tip, depresses the rate of elongation of the decapitated root of *Lupinus.*

Data are presented on the rate of inactivation of the eye-stalk substance by aging, and on the concentration effect.

The plant growth hormone will not activate chromatophores of crustaceans as the eye-stalk extract of crustaceans does. Nor have we been able to extend the analogy between the pituitrin-like qualities of the eye-stalk hormone and the plant growth substance by finding a chromatophore-activating factor in pregnancy urine.

It is pointed out that measurements permitted by the conditions of these experiments open the way to the assay of the eye-stalk chromatophore activator, and thus to the quantitative investigation of its origin and properties. They also raise interesting possibilities for the analysis of the growth-hormone effect in plants.

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THE EFFECT OF RADIATION UPON CELL RESPIRATION

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It has long been known that exposure of young organisms to sufficiently large doses of short wave length radiation inhibits subsequent growth and development. It is the purpose of this paper to consider a possible seat of attack by radiation.

Since the primary effect of radiation is to ionize indiscriminately the atoms and molecules in the permeated medium, it is not a necessary—nor even probable—assumption that any one cell process is affected specifically to give the end result. However, it is profitable to investigate separately the reactions of different physiological processes in an attempt to find how organisms are affected by radiation. For the present study, cell respiration has been chosen. Since growth and development are so markedly affected by radiation, it is but reasonable to expect that the energy-releasing mechanism might be a seat of attack; certainly if respiration were profoundly altered, there would be a pronounced effect upon growth and development.

Gottschalk and Nonnenbruch (1923), Adler (1930), and Crabtree (1932) have reported that respiration in a variety of plant and animal organisms is impeded by X-rays and gamma rays. Usually there is a latent period of several hours before the effect is noticed.

Wels (1924) found no effect of X-rays upon respiration in blood cells, chopped muscle and yeast. Hubert (1929) found no effect upon glycolysis in chick embryos and rat retinæ; he considers glycolysis the most sensitive of the energy-releasing mechanisms.

Redfield and Bright (1922) irradiated dry radish seeds with emanation, and after 2 days moistened them and measured the CO_2 production. Growth in the irradiated seedlings was inhibited while CO_2 production was increased.

Francis (1934) found that there are parallel decreases in fresh weight and CO_2 production in the irradiated wheat seedling.

From the literature cited, it is clear that there is no consistent agreement as to whether radiation affects cell respiration directly. The work of Adler (1930) and Crabtree (1932), showing a latent period of several hours before respiration is decreased, suggests that the effect upon respiration is secondary. From the parallel decreases in growth and respiration which Francis (1934) demonstrated, it may be surmised that respiration simply follows growth which is primarily reduced. However, positive evidence for this hypothesis is lacking, and the converse may obtain.

In the present paper, the questions considered are: (*a*) whether respiration, per unit mass of protoplasm, in the wheat seedling is affected by radiation in doses sufficient to retard growth markedly, i.e., whether growth and respiration are inhibited in parallel amounts; (*b*) whether the respiration of certain marine eggs is impeded when marked developmental anomalies are produced, and (*c*) whether the effects on growth and development are dependent upon a primary reduction in respiration, or whether the depressed respiration depends upon a reduction in growth.

Wheat Seedlings

Methods

Wheat seeds were soaked for an hour and sprouted in a moist chamber kept in a dark incubator at 26° C. At the end of 18 hours, when the embryo seedlings had just broken through the seed coat, the organisms were selected for uniformity, placed upon a wet filter paper in a celluloid tray, and irradiated.

The source of gamma rays was a four-gram radium pack which has been fully described by Failla (1928). The seedlings were exposed for different periods up to 3 hours at a distance of 5.8 cm. from the radiation source. The radiation was filtered by a minimum of 0.35 mm. of platinum, 1.5 mm. of brass, and 6 mm. of bakelite. Hence the primary radiation consisted almost entirely of gamma rays.

The source of X-rays was a Coolidge tube with a tungsten water-cooled target. The machine was operated at 200 kv. and 32 ma. The seedlings were placed 35 cm. from the target, and covered with the filter; the X-rays passed through 0.15 mm. of copper, 0.5 mm. of aluminum, and 2.5 mm. of celluloid, in order. The intensity of radiation reaching the seedlings, under these conditions, was 226 r. per minute. Exposures of 2.5, 5, 7.5, and 10 minutes were made.

As soon as treated, the seedlings were placed in moist chambers and put either into the incubator at 26° C. or into an ice-box at 6° C. At this latter temperature, the seedlings did not grow to any appreciable extent; when removed to a higher temperature, growth proceeded without any ill effect resulting from the exposure to 6° (see also Henshaw and Francis, 1933).

Respiration was determined ½, 4, 6, 24, 48, and 72 hours after irradiation, in several series of experiments totaling about 70 measurements for each dose of X-rays used, and conducted during every season of the year.

The oxygen consumption was determined by placing from 5 to 10 seedlings, depending upon size, in Warburg respiration vessels of capacities of about 14 cc. All respiration experiments were made at 26° C., usually over a period of an hour with readings taken at 10-minute intervals. At the end of this time, the seedlings were carefully removed from the grains, blotted, and weighed at once. Oxygen consumption was calculated on the basis of fresh weight and per seedling.

Results

In Table I are shown the data for a typical experiment, in which fresh weight and oxygen consumption were determined 24 hours after irradiation. Column 6 shows that the reduction in growth, caused by irradiation, is almost exactly paralleled by the reduction in respiration per seedling. Under all conditions investigated, except when the oxygen consumption was measured ½–6 hours after irradiation, the results were the same: irradiation sufficient to impede growth considerably has no effect upon the respiration of the organism *when account is taken of the mass of growing tissue.* The deviations of all the experiments, except those of the ½ to 6-hour period, are summarized in Table II. The oxygen consumption per gram of fresh weight per hour was calculated for each set of seedlings; this respiratory index of the control was taken as 100 per cent, and the indices of the irradiated seedlings were expressed as percentages of the control. The table shows the distribution of the data about the modal 100 per cent. In other words, the oxygen consumption per unit mass of tissue is the same in control and irradiated seedlings, whatever the conditions of the experiment, with the exception noted above when the respiratory index is greater in the irradiated seedlings.

A direct attack of radiation upon the respiratory mechanism is precluded by the fact that measurements of oxygen consumption made during the first 6 hours after irradiation showed little change in the rate of O_2 consumption of the treated seedlings as compared to the controls; this is based upon the respiratory rate per seedling. Yet at the end of 6 hours, there is a detectable difference in the growth of the control and irradiated seedlings, as shown in Table III. *Growth has* *been impeded before respiration*, and this growth inhibition, therefore, can not depend upon a primary reduction in the respiratory rate. If the radiation were to inactivate or destroy the respiration catalysts within 6 hours after exposure, the method could not fail to detect a decrease in the respiratory rate of the irradiated seedlings. Such a decrease was not observed. The results of a typical series of experiments are shown in Table III.

TABLE I

The effect of radiation upon respiration and fresh weight of wheat seedlings. Measured 24 hours after irradiation. Kept at 26° C.

Dose	Respiration		Fresh weight		Resp./weight
0	*$mm.^3$ O_2/hour*	*% control*	*grams*	*% control*	*per cent*
Control	163		0.090		
	193		0.130		
	177		0.095		
	205		0.125		
	173		0.100		
	227		0.155		
	228		0.190		
	213		0.150		
	180		0.160		
Mean	195	100	0.133	100	100
2.5 min.	143	87	0.085	94	92
	142	79	0.080	61	128
565 r.	139	78	0.070	73	107
	163	80	0.100	80	100
	142	82	0.090	90	91
	175	77	0.140	90	81
	188	82	0.140	74	110
	183	86	0.125	83	103
	167	93	0.140	87	107
	160	83	0.109	81	102
5 min.	119	73	0.070	77	95
	152	85	0.095	73	116
1130 r.	130	73	0.065	69	106
	143	70	0.085	68	102
	134	77	0.070	70	110
	158	67	0.120	77	87
	179	78	0.125	66	118
	199	93	0.105	70	132
	114	63	0.140	87	72
	147	75	0.097	73	103

Table I—*Continued*

Dose	Respiration		Fresh weight		Resp./weight
	mm.³ O_2/hour	*% control*	*grams*	*% control*	*per cent*
7.5 min.	97	60	0.065	72	83
	165	64	0.075	57	112
1695 r.	124	62	0.065	69	90
	110	63	0.080	64	98
	129	66	—	—	—
	143	61	0.090	59	103
	161	71	0.115	60	118
	138	64	0.100	67	95
	127	70	0.130	80	87
Mean	133	68	0.090	68	99+
10 min.	101	62	0.055	61	101
	123	63	0.065	50	126
2260 r.	134	75	0.065	68	110
	131	63	0.074	59	106
	106	61	0.060	60	101
	154	65	0.090	59	110
	157	69	0.100	53	130
	137	63	0.080	53	119
	116	64	0.120	75	85
Mean	128	66	0.079	60	110

Since the respiratory rate is nearly constant in both control and irradiated seedlings during the first 6 hours after treatment, while there is a difference in the growth of the two sets, it follows that the respiratory index of the irradiated seedlings will be somewhat higher than that of the controls at this time. This is evidently the fact, as shown in Table III. The following day, the respiratory index is the same for both control and irradiated seedlings, which suggests that respiration simply follows growth.

An indirect attack of radiation upon the respiratory mechanism is improbable, as shown by the ice-box series of experiments. Seedlings were placed in moist chambers as soon as irradiated (as described above); they were at once put into the ice-box at 6° C. Controls were also kept at this temperature. These seedlings did not grow, and hence the control and irradiated seedlings were of the same size at the end of 48 hours in the ice-box. When the O_2 consumption was determined, at 26° C., up to 48 hours after irradiation, it was found to be the same in control and irradiated seedlings whether based on fresh weight or per seedling. Under the conditions of the experiment, there had been neither a direct nor an indirect attack by radiation upon the respiratory mechanism. When the seedlings were subsequently put into an incubator at 26° C., the amounts of growth and respiratory inhibition obtained after 24 hours were identical with those obtained when the seedlings were put directly into the incubator (the latter are shown in Table I). In other words, there had been neither recovery nor aggravation of radiation effect during the period in the ice-box.

TABLE II

The effect of X- and gamma radiations upon respiration of wheat seedlings. Oxygen consumption per gram fresh weight per hour in control is 100 per cent.

Two and one-half minutes exposure (565 r.)								
Class..........	76–82	83–89	90–96	97–103	104–110	111–117	118–124	125+
Frequency.....	2	9	11	16	12	3	4	2
Five minutes exposure (1130 r.)								
Class..........	76–82	83–89	90–96	97–103	104–110	111–117	118–124	125+
Frequency.....	3	9	8	15	7	8	5	3
Seven and one-half minutes exposure (1695 r.)								
Class..........	76–82	83–89	90–96	97–103	104–110	111–117	118–124	125+
Frequency.....	7	6	11	20	10	2	6	3
Ten minutes exposure (2260 r.)								
Class..........	76–82	83–89	90–96	97–103	104–110	111–117	118–124	125+
Frequency.....	4	8	5	10	8	6	8	4

Mean values: $2\frac{1}{2}$ minutes, 99.3 per cent; 5 minutes, 103.6 per cent;
$7\frac{1}{2}$ minutes, 99.7 per cent; 10 minutes, 103.0 per cent.

MARINE EGGS

Methods

The eggs of *Arbacia* and *Chætopterus* were obtained by the methods described by Whitaker (1933). After several washings in sea water the pooled eggs of several females, in each case, were concentrated to about 1 volume in 40 of sea water. Twelve milliliters of the egg suspension were placed in a Stender dish and irradiated for 30, 45, or 60 minutes (21,600, 32,400, or 43,200 r.). In the case of *Arbacia* eggs, the smallest dose given before fertilization was sufficient to delay the first cleavage to twice its normal time (Henshaw, 1932), and resulted in 100 per cent deaths within 48 hours.

Eggs were fertilized both before and after irradiation. Unfertilized irradiated eggs were fertilized at different times after irradiation. In the fertilized series, irradiation began 15 minutes after fertilization, since the eggs are more radiosensitive at 15 minutes than at 10 or 20, as judged by subsequent development (Miss Rusch, unpublished).

A Coolidge tungsten target air-cooled tube was operated at 120 kv. and 5 ma.; the only filter used was a heavy paper which served to protect the eggs from the heat of the tube. The distance from the target to the eggs was 20 cm. Under these conditions, ionization measurements showed an intensity of 720 r. per minute. The depth of water in the dishes was constant throughout the series and the temperature of the material nearly constant.

TABLE III

The effect of X-rays upon the oxygen consumption of wheat seedlings. Measured during the first 6 hours after irradiation. 26° C. Dose of X-rays 1130 r.

Experiment	1	2	3	4	5
Weighed, hours after irradiation	3	4	5	5	5
Control:					
O$_2$/seedling/hour (hour before weighing), mm.^{3}	12.3	14.8	19.2	16.3	17.1
Fresh weight, grams	0.00335	0.00416	0.00566	0.00422	0.00533
O$_2$/gm./hour, mm.^{3}	3690	3556	3400	3852	3202
Resp. index, per cent	100	100	100	100	100
Irradiated:					
O$_2$/seedling/hour (hour before weighing), mm.^{3}	10.1	14.6	18.2	16.3	16.0
Fresh weight, grams	0.00240	0.00353	0.00477	0.00355	0.00455
O$_2$/gm./hour, mm.^{3}	4200	4128	3808	4548	3506
Resp. index, per cent control	114	116	112	118	109
Number of seedlings	30	30	45	45	45

Respiration was measured by the Barcroft-Warburg manometric method, using vessels of 5.3 cc. capacity, with a fluid volume of 1.7 ml. The oxygen consumption was determined over a period of an hour, at 25° C.

The influence of irradiation upon the increase in oxygen consumption which occurs when methylene blue is added to a suspension of *Arbacia* or *Chætopterus* eggs was determined. Grübler's methylene blue was added to give a final concentration of 0.005 per cent.

The volume of eggs was determined by placing the washings of the respiration vessels in vaccine tubes and centrifuging until the egg mass had attained a constant volume; 5 minutes at a given high speed seemed to suffice. The method gives volumes which are too high, and consequently the respiration per unit mass, as calculated from these data, will be less than the true absolute values (Rubinstein and Gerard, 1933). However, since control and irradiated eggs were centrifuged opposite to each other, the figures obtained are comparable among themselves.

Table IV

The effect of X-rays upon respiration of Arbacia *eggs.* Oxygen consumption per hour per ten cubic millimeters of eggs. Egg concentration *ca.* 1 : 40. 25° C. Dose 21,600 roentgens.

Exp.	Control unfertilized	Irrad. unfertilized	Control fertilized	Irrad. fertilized	Irrad. unf. fert. immed.	Irrad. unf. fert. later
1	0.24	0.21	0.55	0.35	—	0.23
2	0.27	0.26	0.46	0.46	0.57	—
3	0.32	0.28	0.38	0.41	—	0.50
4	0.28	0.34	0.38	0.37	0.48	—
5	0.19	0.11	0.36	0.33	—	0.43
6	0.33	0.27	0.37	0.39	0.37	—
7	0.31	0.30	0.40	0.40	0.41	—
8	0.23	0.20	0.32	0.36	—	0.40
9	0.21	0.18	0.40	0.41	0.29	—
10	—	—	0.33	0.30	—	—
11	0.17	0.22	0.26	0.23	0.34	—
12	0.13	0.13	0.30	0.24	—	0.42
13	0.17	0.12	0.37	0.38	0.22	—
14	0.21	0.21	0.41	0.36	0.42	—
15	0.27	0.24	0.48	0.46	—	0.47
16	0.22	0.22	0.55	0.53	—	0.52
17	0.18	0.25	0.45	0.38	0.41	—
18	0.23	0.23	0.30	0.38	—	—
Av.	0.23	0.22	0.39	0.38	0.39	0.42

Results

In Table IV are summarized the results of 18 experiments with *Arbacia eggs.* It will be seen that large doses of X-rays sufficient to hamper the early development and to kill the larvæ before they reach the pluteus stage have no effect upon the O_2 uptake of unfertilized or cleaving eggs. The smallest dose (21,600 r.) was doubled and tripled without any effect upon the respiration, although the cleavages became more abnormal.

Many unequal blastomeres were seen; three-cell stages were frequent; the first cleavage was delayed to twice its normal time in the series receiving the smallest dose; the largest dose (64,800 r.) prevented cleavage entirely, and yet the respiration was uninfluenced.

All measurements of respiration were made within 6 hours of irradiation. This should detect any direct effect that irradiation might have upon cell respiration.

TABLE V

The effect of X-rays upon normal and methylene blue catalyzed oxygen consumption of Arbacia *eggs.* Oxygen consumption per hour per 10 cubic millimeters of eggs. Egg concentration *ca.* 1 : 40. Temperature, 25° C. Mb. 0.005 per cent. X-ray dose 21,600 r.

Control		Irradiated		Control		Irradiated	
Unfert.	Unf. + Mb.	Unfert.	Unf. + Mb.	Fert.	Fert. + Mb.	Fert.	Fert. + Mb.
0.23	0.21	0.23	0.23	0.30	0.50	0.38	0.43
—	—	—	—	0.29	0.55	0.23	0.40
0.11	0.14	0.16	0.26	0.27	0.39	0.28	0.43
0.13	0.24	0.19	0.25	0.30	0.42	0.31	0.50
0.18	0.22	0.19	0.24	0.30	0.34	0.35	0.35
—	—	—	—	0.35	0.45	0.31	0.41
—	—	—	—	0.33	0.39	0.28	0.36
—	—	—	—	0.36	0.39	0.34	0.43
0.16 Mean	0.20	0.19	0.24	0.31	0.43	0.31	0.41

The results of the methylene blue series are given in Table V. Most of these experiments were done with fertilized eggs, as fertilized eggs are more sensitive (Packard, 1933). Comparison of the average values shows that irradiation of eggs and zygotes does not influence the rate of oxygen consumption under normal or methylene blue catalyzed conditions. The fact that the increase in O_2 consumption caused by methylene blue is not affected by X-rays probably means that the substrate-activating enzymes are not influenced by X-rays.

The results observed with the eggs and zygotes of *Arbacia* are paralleled in the eggs and zygotes of *Chætopterus,* as shown in Table VI. There is a considerable variation from experiment to experiment which may be caused by the fact that the eggs were collected near the end of the spawning season. However, the averages, which are close to those reported by Whitaker (1933), show that radiation does not inhibit respiration while it does alter profoundly the course of develop-

TABLE VI

The effect of X-rays upon normal and methylene blue catalyzed oxygen consumption of Chætopterus *eggs.* Oxygen consumption per hour per 10 cu. mm. of egg volume. Egg concentration *ca.* 1 : 40. Temperature, 25° C. Mb. 0.005 per cent. Dose 32,400 roentgens.

Control		Irradiated		Control		Irradiated	
Unfert.	Unf. + Mb.	Unfert.	Unf. + Mb.	Fert.	Fert. + Mb.	Fert.	Fert. + Mb.
1.1	1.5	1.5	2.7	1.6	2.7	2.3	3.0
2.2	3.5	1.3	2.5	1.4	2.1	1.1	1.7
2.0	3.1	2.4	3.7	(7.6)*	(5.6)*	2.1	2.1
—	2.0	1.7	2.7	—	2.7	2.3	2.0
2.1	1.5	1.1	1.2	1.2	1.7	1.0	1.4
1.9	2.7	2.7	3.3	1.6	1.0	(0.3)*	(0.70)*
1.9	2.4	1.8	2.7	1.5	2.0	1.8	2.2

* Not averaged.

ment. In the case of *Chætopterus* eggs there is no cleavage delay caused by sublethal doses of X-rays. However, the large doses given do cause abnormal cleavages—different-sized blastomeres, three-cell stages, many larvæ never reaching the free swimming stage and few passing it.

The increase in respiratory rate, after fertilization, is the same in both control and irradiated eggs, as shown in Table VII. There is a large variation from experiment to experiment which may vitiate the

TABLE VII

The increase in respiratory rate, with time, in Arbacia *zygotes.* Oxygen consumption per hour per ten cubic millimeters of egg volume.

Resp. 1.6 ± 0.4 hours after fertilization		Resp. 4.5 ± 1 hours after fertilization		Increase in percentage	
Control	Irradiated	Control	Irradiated	Control	Irradiated
				per cent	*per cent*
0.45	0.38	0.55	0.53	22	39
0.41	0.36	0.48	0.46	17	28
0.37	0.38	0.51	0.46	38	21
0.26	0.23	0.30	0.24	15	5
0.38	0.37	0.38	0.41	0	8
Av. 0.37	0.35	0.44	0.42	19	20

conclusion drawn, but it seems probable that radiation does not prevent the normal rate of increase in O_2 consumption.

Discussion

It is the purpose of this paper to consider respiration as a possible seat of radiation attack in growth inhibition. Since both growth and respiration are cut down in the same degree by exposure to radiation, the answer is to be had by determining which of these inhibitions is dependent upon the other—by separating cause and effect.

If growth inhibition be caused by respiratory inhibition, then the effect of radiation upon respiration must be caused by inactivation of catalysts already present or by a decreased production of new catalysts which would prevent an increasing rate of O_2 consumption, and therefore further growth. A definite basal rate of respiration must be necessary for growth, and if the respiration be retarded by a depletion of catalysts, this basal rate could not be maintained, and growth would be hindered. This possibility of a primary reduction in respiration is ruled out by the following facts, established experimentally: (*a*) growth in the wheat seedling is impeded before the respiratory rate is altered, (*b*) when growth is arrested by low temperature, no respiratory changes, per seedling, appear, (*c*) in the marine eggs, developmental anomalies appear in spite of the fact that the respiratory rate increases equally in irradiated and control eggs.

The interpretation of the ice-box experiment is precarious because of the fact that the cell processes are slowed down to the point at which no growth occurs. There is the possibility that there is some reaction from irradiation involving the respiratory catalysts which does not proceed at 6° C., while at higher temperatures it does go on and impede respiration. This seems rather improbable since seedlings are equally radio-sensitive whether irradiated and grown at 19° or 26° C., which would mean that the postulated reaction has a temperature coefficient of 1.

The possibility that the seat of radiation attack is the mechanism synthesizing new respiration catalysts must be considered. Apparently the production of new catalysts is in abeyance during the early stages of seedling growth with which we are dealing. This is shown by the rapid decrease in respiratory index with time, and by the direct determinations which show that the respiratory rate per seedling is nearly constant during the first 6 hours after irradiation, although there is a demonstrable difference in the growth of control and irradiated seedlings. This consideration, together with the unaltered increase in

O_2 uptake of irradiated eggs, all but rules out the synthetic mechanism as the seat of radiation attack.

Since Miss Francis found that radiation did not influence the CO_2 production per unit mass in the wheat seedling (with the exception noted), and I have shown that the index of O_2 consumption is unaltered (with the same exception), it follows that radiation does not change the R. Q., i.e., the type of metabolism is unchanged.

Nemenow (1925) and Isaacs (1932) have set forth arguments for the hypothesis that radiation ages the cell. From the present results an objection may be raised. The respiratory index is a function of age, and shows a progressive decrease as the organism grows older. Radiation does not alter this index (with the exception noted), and therefore does not age the cell as judged by this criterion.

From the work reported in this paper, it seems probable that the respiratory changes are dependent upon the morphological changes observed. In the case of the wheat seedling the amount of oxygen consumed by the irradiated seedling is less simply because there is less tissue to respire, not because of any impairment in the respiratory mechanism itself. In the marine eggs, there are profound alterations in morphology without any change in respiration. In the eggs, the morphological changes observed are in cell division, subsequent monster formation and death; in the seedling the change is in growth.

These experiments do not, of course, prove that growth and development are independent of respiration. Development must depend upon several factors, one or some of which are radio-sensitive to a degree that development is interfered with by doses of radiation which have no effect upon respiration.

I wish to acknowledge my indebtedness to Dr. G. Failla and Dr. P. S. Henshaw, of Memorial Hospital, for criticisms. Dr. R. W. Gerard, of the University of Chicago, also offered valuable advice.

SUMMARY

Wheat seedlings were irradiated with X-rays and gamma rays in such doses as to impede growth by 40 per cent, as measured 24 hours after treatment. Various experimental conditions were maintained.

The oxygen consumptions of the control and irradiated seedlings were determined at different intervals after irradiation, using the Barcroft-Warburg manometric method.

Under all conditions investigated, except when the respiration was determined within 6 hours after irradiation, the results were the same—oxygen consumption per gram of growing tissue is not affected by doses of radiation sufficient to inhibit growth by as much as 40 per cent.

Growth is inhibited before the respiratory rate changes. This is shown by the measurements made during the first 6 hours after irradiation, when the respiratory index is higher in the irradiated than in the control seedlings. This precludes a direct attack of radiation upon the respiratory mechanism.

When growth is stopped by exposure of seedlings to 6° C., there is no observable difference in the respiratory rates of the control and irradiated seedlings, per seedling, even after 48 hours. This makes improbable any attack of radiation upon the respiratory mechanism itself.

There is no observable change in the respiratory rate, per seedling, until the growth of the irradiated seedling lags behind that of the control; then the respiration per unit mass becomes the same in both.

The eggs of *Arbacia* and *Chætopterus* were given large doses of X-rays sufficient to hamper their early development, produce abnormal cleavages, and cause early death.

The respiration of the irradiated eggs, measured by the Barcroft-Warburg method, was not affected by the doses of X-rays given. The increase in oxygen uptake under the influence of methylene blue was also unchanged by X-rays.

The rate of increase in respiration of control and irradiated eggs, with time, is similar. Developmental anomalies appear before the respiratory rate is altered, and do not, therefore, depend upon respiratory impairment.

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OBSERVATIONS ON THE EXPERIMENTAL ADAPTATION OF CERTAIN FRESH-WATER CILIATES TO SEA WATER[1]

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The taxonomic literature of protozoölogy includes many references to species of Protozoa which are said to be found in both salt and fresh water. Especially is this true for the Ciliata. Kudo (1931) lists numerous species of this class as found in both habitats. Among them are two species of *Euplotes, E. patella* and *E. charon.* Having made considerable study of *Euplotes,* both fresh-water and marine forms, and having failed to find either of these two species in sea water, it occurred to me that a worthwhile experiment might be carried out to determine whether or not *Euplotes patella,* a common fresh-water form, might be gradually adapted to live in sea water. I have not been able to find the record of any such experiment with ciliates. The most outstanding experiment of this sort with any protozoan is that made by Zuelzer (1907) with Amœba.

The results recorded below represent the findings of experiments extending over about nine months.

Two problems are involved in such a study. First, will the organisms survive in sea water; and secondly, if they will survive, what effects have the changed conditions had on the normal metabolic and reproductive functions?

The materials used were mixed cultures of many Protozoa, the most numerous being *Euplotes patella, Paramœcium caudatum,* and *Spirostomum ambiguum,* with lesser numbers of other forms, developed according to the culture methods of Turner (1930). While numerous different experiments were attempted with varying degrees of success, those in which the animals were carried into the highest concentrations of sea water were conducted as follows: To 9 cc. of the culture fluid containing large numbers of the different animals was added 1 cc. of filtered sea water. To this were added two or three grains of boiled wheat. Each day there was added 1 cc. of sea water until a 50 per cent concentration of sea water was reached. While this gradually increased the actual concentration, it is readily seen that the daily rate of increase gradually decreased. Experience proved that after the cultures reached a sea water concentration of 50 per cent, it was better to increase the interval to two days between additions of more sea water.

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[1] The sea water used was obtained at Cape Foulweather, Oregon, six miles north of the mouth of the Yaquina River, the nearest stream of any importance. The water was filtered through filter paper and stored in a large carboy until used. The total salt content was approximately 31.8 parts per thousand.

In some preliminary experiments it was found that introduction into 10 per cent sea water had little if any effect on the general activities of the three Protozoa mentioned, while a sudden change from the original culture to one of 20 per cent sea water had very pronounced effects. All of the Protozoa were more or less paralyzed at once and settled to the bottom of the container. In a short time—five minutes—all but *Euplotes* were dead. *Euplotes* apparently were able to withstand the sudden change in salt content of the medium better than the others. While partially paralyzed at first, within an hour nearly all had recovered and appeared to move about in normal fashion. Within a few hours they had collected about the grains of wheat and a day or so later fission was noticed and the colony flourished.

In the regular experimental cultures where the sea water content was increased by the daily addition of 1 cc. of sea water, all three kinds of the Protozoa lived in concentrations higher than 20 per cent. With the gradual increase above 20 per cent there was a coincident decrease in the number of *Paramœcium* and *Spirostomum,* until in cultures of 60 per cent or more few if any survived. Those that could be found appeared small and resembled starved individuals. The *Euplotes* were able to survive further additions of sea water and in fact dividing individuals were found in cultures of 66 per cent sea water. In many of the experiments it was possible to carry numerous *Euplotes* up into concentrations of 80 per cent to 85 per cent sea water, in which they would live for several days. In these concentrations no divisions were observed and the number of individuals gradually decreased until all had died. In one experiment one individual was kept in 100 per cent sea water for several days. I have not been able to repeat this result in more than a dozen attempts.

The adaptation to these higher concentrations of sea water could be accomplished only by very gradual changes. Three weeks were involved in bringing some of the cultures up to 80 per cent sea water in which the animals lived for a few days and then died.

Interesting observations were made on the effects of the different amounts of sea water on the contractile vacuole both of *Euplotes* and *Paramœcium.* While there were no visible effects on the general behavior of the two forms, when introduced into 10 per cent sea water, closer observations on the activity of the contractile vacuole disclosed that the function of this organelle had been modified. By using the method described by Taylor (1923) for holding *Euplotes* under a surface tension film of water in a moist chamber the rate of pulsation of the vacuoles could be carefully studied. In fact the pulsations nearly if not entirely ceased. Specimens have been kept under observation for more than an hour without any discharge of the vacuoles which remained in the various diastolic phases at which they had been caught when introduced into the 10 per cent sea water.

If *Euplotes* were brought into the higher concentrations of sea water rapidly with the increases above 50 per cent taking place at intervals of less than two days, it was found that when a concentration of 65 per cent sea water was reached there appeared in the animals a large vacuole in the usual location of the contractile vacuole. This did not discharge but on the contrary increased in size and became so large as to make the whole individual spheroidal, with a thin layer of protoplasm surrounding the relatively enormous vacuole. This condition appeared almost as an epidemic and within a few hours after its onset all of the animals in that culture died. These results have been repeated many times with the same results although there might be some variation in the relation of its appearance to the sea water concentration of the culture. Sometimes the enlarged vacuole did not appear until the culture had become as much as 75 per cent sea water. It would seem that the increased concentration of the sea water caused a change in the ectoplasm of the organisms which prevented the discharge of the contractile vacuole. Just what this change is and to just what it is due have not been determined.

While it has not been possible to draw any definite conclusions regarding the question as to whether or not the species of *Euplotes* considered is found both in fresh water and the sea, some facts have been brought out. In the first place it has not been possible in the experiments attempted to develop a flourishing culture of *Euplotes patella* in concentrations of sea water higher than 66 per cent.

It has been possible to get a few individuals to live in sea water of almost 100 per cent for a short time, but in no case were divisions observed in concentrations above 66 per cent and in that only rarely. It is possible to conceive of the change from fresh to salt water as being so slow in the mouths of streams and in brackish swamps that *Euplotes* might become adapted and flourish, but so far we have no direct evidence for that taking place. The older works of Kent (1881) and others describe *Euplotes patella* as living under both conditions, but their illustrations resemble much more the appearance of some of the species of *Euplotes* that are known to live in salt water. (Cf. *E. salina* and *E. minuta,* Yocom, 1930.) Like these marine species the forms pictured lack the wide triangular cytostome of the common fresh-water *Euplotes patella.* Those of us who have recently studied the salt water representatives of the genus have not seen *Euplotes patella* either in the Atlantic or Pacific. If they were at all common in that environment as they are in fresh water it seems doubtful that they would have been completely overlooked.

In answer to the question as to whether or not *Euplotes* will survive and become adapted in sea water, we will have to say that on the information gained from these experiments it seems doubtful. The experiments have brought out the fact that very marked and detrimental changes in metabolism are caused by the increasing concentration of sea water in the cultures. While *Euplotes* is better able to withstand the changes toward a sea-water habitat than either *Paramœcium* or *Spirostomum,* few were able to survive concentrations greater than 75 per cent.

An interesting study will be to attempt the reciprocal experiment of bringing marine *Euplotes* into fresh water. This will be taken up when the opportunity is afforded.

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IMPATERNATE FEMALES IN HABROBRACON[1]

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Introduction

The parasitic wasp *Habrobracon juglandis* (Ashmead), in common with the majority of insects of the order Hymenoptera, regularly produces females from fertilized eggs, males from unfertilized eggs. P. W. Whiting (1924) has reported the occurrence of several exceptional females coming from virgin mothers. Occasional production of females from unfertilized eggs (thelytoky) had previously been recorded in other Hymenoptera which normally produce males from unfertilized eggs (arrhenotoky). It was not observed again in *Habrobracon* until the summer of 1930. At that time a virgin female homozygous for the recessive gene tapering, *ta* (antennæ), was crossed to a male that was spread, *sp* (wings), for the purpose of testing possible linkage between the two mutant characters. Seven F_1 females from the cross were isolated prior to their eclosion and bred as virgins. They produced 12 female offspring and 307 males. Impaternate females (females from unfertilized eggs) have occurred in the F_2 generation from all subsequent crosses where tapering females have been mated to males of other stocks.

An attempt has been made to determine (*a*) the types of crosses which produce impaternate females in the F_2; (*b*) the various ratios in which these impaternate females occur; (*c*) the chromosome composition of impaternate females as indicated by their mutant characters and those of their offspring.

A stock of *Habrobracon* homozygous for the dominant trait Minnesota-yellow, *My* (base of antennæ), was derived by P. W. Whiting from wasps sent from Minneapolis by Royal N. Chapman in 1929. The stock has been inbred since its derivation. Lysbeth H. Benkert discovered in it several mutations including the two recessives, tapering and shot-veins, *sv* (wing veins). These and other mutant characters used in this work have been described by P. W. Whiting (1932*a*,

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[1] The research here reported has been carried on at the University of Pittsburgh and at the Marine Biological Laboratory, Woods Hole, Massachusetts. It has been aided by grants from the Committee on Effects of Radiation on Living Organisms (National Research Council) to Dr. P. W. Whiting who has suggested the problem and directed the work.

1934*b*). Both tapering and shot-veins stocks have been inbred since their derivation.

The mutant character tapering affects the number and shape of the antennal segments. Normally there are from 14 to 16 of these in the female and from 21 to 24 in the male. In tapering individuals they are reduced in number and show considerable fusion and distortion, especially toward the distal end of the antenna. Male antennæ are likely to be more abnormal than female, so that antennal length is often approximately the same in the two sexes.

The factor tapering is unique in *Habrobracon* for the frequency with which it reverts to type. A stock known as reverted tapering, *reta,* which has the same residual heredity as tapering but lacks the mutant character, has been derived and used in certain of the experiments. It has the following advantages over tapering. (*a*) The facility with which the sexes can be distinguished is increased in wasps with normal antennæ. (*b*) There is a possibility of observing the segregation of other mutant traits affecting antennæ which would be obscured by the presence of tapering. (*c*) Any decrease in viability due to the factor tapering either when it occurs alone or in combination with other recessives is avoided.

Shot-veins females used in crosses have been peculiarly productive of F_2 mosaic males, as shown in extensive experiments by Raymond J. Greb (1933*a*, 1933*b*), but have rarely produced impaternate females in F_2. On the contrary, tapering and reverted tapering females, while regularly producing impaternate F_2 females, only rarely give rise to mosaic males. Fifty-eight impaternate females and only one mosaic male were included among 8083 F_2 individuals from crosses of tapering and reverted tapering females to orange, *o* (eyes), males from stock 11-*o*, or to crescent, *cr* (eyes), males.

Occurrence of Impaternate Females

Crosses Producing F_2 Impaternate Females

Outcrossing Tapering and Reverted Tapering Females.—Impaternate females occur in the F_2 generation, as daughters of virgin F_1 females. In the majority of cases the latter were isolated from crosses of tapering females by males carrying various mutant traits. Ratios of F_2 females to total F_2 population were calculated for each type of male used. These ratios differed significantly as tested by the x^2 method ($P = .0002$), indicating that the ratio of impaternate females produced in the F_2 is influenced by the constitution of the male used in the original cross as well as that of the female. Two crosses were made of tapering females to type males from two different wild stocks.

The ratio of F_2 females to total F_2 population from both these crosses was significantly higher than the average ratio from crosses of tapering females to mutant males ($P = .037$, $P = .054$). The percentage of F_2 females in total F_2 population from all crosses of tapering females to mutant and to wild-type males averaged .724.

In two experiments tapering females were crossed to the same males as reverted tapering females. In the first both types of females were crossed to orange males from stock 11-*o*; in the second both types of females were crossed to crescent males. In neither case was there any significant difference between the ratios of impaternate females produced in the F_2 from crosses of tapering and those of reverted tapering females. Tested by the x^2 method the probability that these ratios were not statistically different was .924 for crosses with orange males, and .438 for crosses with crescent males.

TABLE I

F_2 from Outcrosses of Tapering (ta) and Reverted Tapering (reta) Females

Crosses made	F_1 virgins	F_1 virgins producing ♀♀	F_2			Probability of agreement with total % ♀♀ from *ta* ♀♀
			♂♂	♀♀	% ♀♀	
ta ♀♀ by *o. sp*	119	16	2,739	30	1.083	.037
sp	8	3	106	4	3.636	.0008
wh	45	12	1,965	22	1.107	.054
wh. st	258	40	13,498	90	.662	.484
d. c. l. n	122	9	2,599	19	.726	.711
11-*o*	156	24	9,284	48	.514	.031
cr	37	5	1,115	10	.889	.528
stock 1	23	3	445	7	1.549	.041
stock 11	1	1	58	2	3.333	.017
Total from *ta* ♀♀	769	113	31,809	232	.724	
reta ♀♀ by 11-*o*	123	23	7,952	42	.525	
cr	35	5	1,407	17	1.194	
Grand total	927	141	41,168	291	.702	

Incidentally the two experiments just described demonstrate again the fact that the constitution of the males used in the parental crosses influences the ratio of females in the F_2. In the case of both tapering and reverted tapering, females mated to crescent males were daughters of sisters of those mated to orange males. In spite of the fact that females were so closely related in the two experiments, the average percentage of F_2 females in total F_2 population was 1.071 from crosses made to crescent males, and only .522 from crosses made to orange males. The probability that these two percentages are not significantly different is only .0009 as tested by x^2.

Data from crosses of tapering females to mutant males, tapering females to wild-type males, and reverted tapering females to mutant males are given in Table I, and the three series of experiments are considered together in the treatment that follows.

Of the 927 F_1 virgins that produced offspring, only 141 produced daughters. The average size of F_2 fraternities not including females was 36.6 individuals, that of fraternities including females was 52.9. The difference seems to indicate simply that all F_1 virgins have the potentiality for thelytokous reproduction but, as expected, those producing the most offspring are the ones most likely to produce one or more daughters.

Table II

Influence of Age of F_1 Females on Ratio of F_2 Impaternate Females

Vials	F_2			Probability of agreement with total % ♀♀
	♂♂	♀♀	% ♀♀	
a	1561	85	5.164	.020
b	1695	71	4.020	.779
c	1508	51	3.271	.260
d	1233	41	3.218	.258
e	785	26	3.205	.347
f and *g*	392	17	4.337	.904
Total	7174	291	3.898	

When a female is to be bred she is placed in a shell vial with several host caterpillars. After three or four days, when the larvæ from her first eggs are about to pupate, she is transferred to a second vial. The transferring is continued regularly during the life of the female and successive vials are lettered alphabetically. Thus the vials cover approximately equal productive periods. To compare the ratio of daughters produced by F_1 females at different ages, counts of progeny in the "*a*" vials from all females which produced impaternate daughters were added together, and the ratio of F_2 females to total population from these vials was calculated. The same was done for succeeding vials. Tested by the x^2 method the ratios differed significantly ($P = .010$). There is a steady decrease in the percentage of F_2 females in the total F_2 population from "*a*" through "*c*" vials. A rise after "*e*" is not statistically significant. Data are given in Table II.

Inbreeding Tapering and Reverted Tapering Females.—F_1 daughters of sibling crosses in both tapering and reverted tapering stocks were isolated and bred as virgins in order to determine whether outcrossing is necessary to the production of F_2 impaternate females. Fifty-nine tapering virgins produced a total of 4641 male offspring and one female. Seventy-nine reverted tapering virgins produced 2415 male offspring and three females, two of the latter appearing in the same fraternity. Females constituted .006 per cent of the F_2 population from these stock crosses. Since this percentage is significantly lower than the .702 per cent obtained from crossing tapering and reverted tapering females to males from other stocks (by x^2, P is less than .0000001), it appears that outcrossing greatly increases the ratio of impaternate females although it is not essential to their production.

Table III

Influence of X-raying F_1 females, from tapering (*ta*) or reverted tapering (*reta*) females crossed with crescent (*cr*) males, on number of progeny and ratio of F_2 impaternate females.

F_1 ♀♀	F_2			
	♂♂	♀♀	Offspring per ♀, per vial	% ♀♀
X-rayed, *ta/cr*, 78	2145	3	6.3	.139
Control, *ta/cr*, 37	1115	10	7.1	.889
Probability that treated and controls do not differ			$<10^{-7}$	.001
X-rayed, *reta/cr*, 84	2129	4	5.2	.188
Control, *reta/cr*, 36	1407	17	9.8	1.194
Probability that treated and controls do not differ			$<10^{-7}$	.0002

X-raying F_1 Females from Outcrosses of Tapering and Reverted Tapering Females.—The percentage of mosaic males produced in the F_2 from crosses of shot-veins females by various mutant males is increased by X-raying the F_1 virgins (Greb, 1933*a*). The same method was employed in two experiments in an attempt to increase the production of impaternate females. In the first tapering females were mated to crescent males; in the second reverted tapering females were mated to the same males. In each case some of the F_1 virgins were X-rayed and their sisters were bred as controls. The dosage used was approximately 2500 R-units, which was sufficient to decrease the fertility of the treated females significantly. The percentage of females among their offspring was also significantly decreased. Data are given in Table III.

This result had not been anticipated. It is known from the data of other investigators that not only the ratio of mosaic males but also the ratio of other irregular types in *Habrobracon,* biparental males (Stancati, unpublished), visible mutations, and lethals, is increased by X-radiation. On the contrary, in this case females treated with X-ray produced less of an irregular type of offspring than their untreated sisters.

Crosses Not Producing F_2 Impaternate Females

Tapering Males by Wild-type Females.—Tapering males were crossed to females from two different wild-type stocks in order to determine whether the thelytokous tendency can be transmitted by them to their daughters. Thirty-eight F_1 virgins were bred and produced 1464 F_2 males and no females.

Shot-veins and Minnesota-yellow Females by Mutant Males.—Since tapering, shot-veins, and Minnesota-yellow stocks are so closely related, it was thought probable that the thelytokous tendency might be present in all. In the course of various linkage tests where shot-veins or Minnesota-yellow females were crossed with mutant males, Lysbeth H. Benkert did discover a number of impaternate females among the F_2 offspring. Accordingly one cross was made of a shot-veins female by a mutant male. Forty-six F_1 virgins were bred and produced 1660 F_2 males and no females. Eight series of crosses were made of Minnesota-yellow females by as many types of mutant males. One hundred and sixty-two F_1 virgins were bred and produced 4929 males and no females.

F_2 Impaternate Females by Brothers.—An effort was made early in the work to determine whether the tendency to thelytoky might be increased by selection. Isolation of virgins is accomplished by placing single pupa cases in gelatin capsules. Since the sexes cannot be distinguished in the larval stage and the pupæ cannot be seen because of the cocoons in the pupal stage, it is necessary to isolate males as well as females. This increases the work involved and makes it impracticable to isolate F_2 impaternate females which constitute less than one per cent of that generation. Such females, therefore, eclose with their brothers and usually have mated before they are taken from the culture. They would then be expected to produce daughters normally from fertilized eggs. Several of them have been found that apparently had not mated since subsequently they produced only sons. None were bred that produced a large number of sons and so few daughters as to suggest thelytokous reproduction. Impaternate females among the offspring of mated impaternates could be detected only if the genetic constitution of parents and offspring were particularly favorable, and no such case was found.

Attempts at selection, therefore, had to be made among the offspring of normal daughters of mated impaternate females. Eighty-one such daughters in the F_3 from tapering females by spread or orange spread males were isolated before eclosion and bred as virgins. They produced 3679 male offspring and no females.

In each of the experiments just described the number of male offspring counted was large enough to show that there was a highly significant difference between these crosses and crosses producing impaternate females. Probabilities that results obtained in these cases were not different from those obtained by outcrossing tapering and reverted tapering females were .001 for crosses of tapering males to type females, .0006 for crosses of shot-veins females by mutant males, less than .0000001 for crosses of Minnesota-yellow females by various mutant males, .0000004 for crosses of F_2 impaternate females by their brothers. Probabilities were calculated by x^2.

CHROMOSOME COMPOSITION OF IMPATERNATE FEMALES

Analysis of Data for Single Loci

Breeding tests show that impaternate females are normal diploids. P. W. Whiting (1924) has explained their production from unfertilized eggs by the assumption that the second maturation division of the oöcyte is suppressed. If this assumption be true and the impaternate females occur among the F_2 from a cross involving mutant characters, their genotypes, as indicated by their appearance and that of their offspring, are of interest in ascertaining whether the first division of the oöcyte from which they were formed was equational or reductional for the locus or loci involved.

If the F_1 female producing an impaternate daughter is heterozygous for a recessive factor and the first oöcyte division is reductional for the locus of that factor, the impaternate daughter will be homozygous for either the recessive or its type allelomorph. If the first oöcyte division is equational, the impaternate daughter will be phenotypically wild-type. Breeding tests are necessary to distinguish impaternate females resulting from an equational division from those receiving the type allelomorph from a reductional division.

An effort was made to secure offspring from all females phenotypically wild-type. Seven type sons from a female should reduce the chance that she was heterozygous to one in one hundred and twenty-eight (2^7), and such a test was considered sufficiently conclusive. According to the results of breeding, all impaternate females which are phenotypically wild-type may be grouped in three classes: (1) those which had no sons or less than seven type sons; (2) those which were proved heterozygous by the production of one or more recessive sons; (3) those which were proved homozygous by the production of seven or more type sons. The first class must be disregarded. The second and third cannot be compared directly since one is more easily tested than the other. If those individuals which produced less than seven sons are subtracted from the second class, it is then comparable to the third.

TABLE IV

Genetic Analysis of F_2 Impaternate Females

Locus	*aa*	*Aa*		*A(A?)*		Total
		7 or more sons	Less than 7 sons	7 or more sons	Less than 7 sons	
ta	63	66	8	26	66	229
o	19	30	2	21	43	115
wh	21	25	2	18	22	88
st	20	27	17	13	11	88
cr	9	6	1	10	8	34
c	3	5	0	4	7	19
l	3	4	0	5	7	19
n	2	4	0	5	8	19
d	2	3	0	5	9	19
sl	1	3	2	0	5	11
sv	3	3	3	2	2	13
wa	1	2	2	0	3	8
el	1	0	1	1	3	6
o^i	0	0	0	0	1	1
k	0	0	0	0	1	1
o^d	0	0	0	1	0	1
tw	0	1	0	0	1	2
gl	0	0	0	0	1	1

Two percentages may be derived for any locus; that of recessives among totals, and that of homozygotes among total wild-type producing seven or more sons. If the latter be multiplied by the percentage of type individuals in the total, then the amount of homozygosis of the recessive and its type allelomorph may be compared directly. It is to be noted, however, that the percentage of recessives among total, which appears from the phenotypes, is more reliable than the percentage of homozygous dominants among total, since the latter can be derived only from genotypes, must depend upon breeding tests, and therefore does not permit the inclusion of all impaternate females.

Data on the phenotypes and genotypes of impaternate females are given in Table IV. The table includes three groups of recessive factors. Females bred to secure the data presented in the third group occurred incidentally in linkage tests made by various workers and hence were obtained in small numbers. The impaternate females included in the second group were all procured from one series of crosses of tapering females by males carrying linked genes on the second chromosome, but occurred in such small numbers that they are scarcely sufficient for establishing ratios. The first group includes the five loci best tested: tapering; orange; white, *wh* (eyes); stumpy, *st* (legs); and crescent. The two percentages described above, as they were derived for these loci, are given in Table V.

TABLE V

Ratios of Homozygous Impaternate Females

Locus	*ta*	*o*	*wh*	*st*	*cr*
% *aa*	27.5	16.5	23.8	22.7	26.4
Probability that % *aa* does not differ from 25	.399	.038	.807	.639	.848
Proportional % *AA*	20.44	34.31	31.85	24.89	44.5
Probability that proportional % *AA* does not differ from 25	.508	.096	.145	.963	.313
Probability that % *aa* and proportional % *AA* do not differ	.266	.039	.464	.850	.442

Since individuals homozygous for a recessive or for its dominant allelomorph are produced as the result of a division reductional for the locus, they are expected to occur in equal numbers. Percentages of homozygosis for the recessive and its allelomorph were compared for each locus by the x^2 method. The probability that there is no significant difference between them is .266 or more except for the orange locus (see Table V).

The case of orange is peculiar and unexplained. The low ratio of homozygous orange individuals (16.5 per cent) would seem to indicate that the orange locus undergoes equational division more frequently than reductional. If this were the case, then few individuals homozygous for the type allelomorph to orange would be expected also. But the proportional percentage of homozygous type individuals to the total is 34.3. Thus there is relatively twice as much homozygosis for the dominant allelomorph to orange as there is for the recessive itself.

Data for orange were collected from three different series of crosses. In the first, tapering females were crossed to orange males from Lancaster stock; in the second tapering females were crossed to orange males from 11-*o* stock; and in the third reverted tapering females were crossed to the same males used in the second. From these three types of crosses percentages of orange among totals were respectively 12, 12.5, and 23.8; percentages of homozygous type among total were respectively 31.9, 34.2, and 35.8; and the probabilities that the two percentages were not statistically different were respectively .157, .031, and .329. Although the probability is not significantly low except in the second type of cross, still it is to be noted that in all cases the difference in percentages is in the same direction; homozygous orange individuals being deficient, homozygous type individuals being in excess. The numbers suggest a factor or factors for increased viability present in the females used in the crosses and closely linked with the type allelomorph to orange.

For the four loci—tapering, white, stumpy, and crescent—the percentage of recessives among total approximates 25. If in the production of impaternate females the second oöcyte division is suppressed, and the first may be equational or reductional with equal chance for any given locus, the number of recessives should be 25 per cent of the total, and the number of homozygous type individuals should be 33.3 per cent of all the wild-type sufficiently tested and 25 per cent of the total. Percentages for the factors given in Table V were tested by the x^2 method for the probability of their concurrence with these theoretical values. If the orange locus be excepted, the probability that the percentages of recessives to totals were not significantly different from 25 was .399 or more, and the probability that the percentages of homozygous type to totals were not significantly different from 25 was .145 or more (see Table V). The case of the orange locus has been discussed previously. For it homozygous recessive individuals make up $25 - 8.5$ per cent and homozygous type individuals make up $25 + 9.3$ per cent of the total. Thus the deficiency of homozygosis in one case is approximately equal to the excess in the other.

It seems clear from study of the data on single loci that random assortment of strands from a tetrad to the poles of the spindle in the first division does not occur. If any strand might pass to the pole with any other, then it would have one chance in six of entering the functional secondary oöcyte with its sister. Homozygosis of any recessive or its type allelomorph would then occur in only 16.6 per cent of the impaternate females, and all factors tested gave percentages of homozygosis well above that figure (allowance being made for the orange recessive). It seems probable, therefore, that each strand may pass to the pole only with a strand that is lateral to it in the tetrad and that sister strands are never diagonal.

The data at hand give ratios approximating one homozygous recessive to two heterozygotes to one homozygous dominant, or homozygosis of one factor in 25 per cent of the cases. The theory that the first division may be equational or reductional with equal chance would fit the data without further assumptions if crossing-over were between diagonal strands only. If this were the case and reductional and equational divisions occur equally frequently at the spindle fiber, each factor would show homozygosis in 25 per cent of the impaternate females.

If crossing-over may be lateral or diagonal with equal chance; that is, if one strand may cross over equally frequently with either of its homologues, as appears to be the case in attached-X chromosomes of *Drosophila* (Anderson, 1925; Emerson and Beadle, 1933), the hypothesis that the first division is as often equational as reductional no longer fits the data; for homozygosis of a factor will then occur in less than 25 per cent of the impaternate females as crossing-over takes place between the marked locus and the spindle fiber, and the frequency of homozygosis will be only 12.5 per cent if the locus is so far away from the attachment that all the tetrads will contain one crossing-over between the two points.

If enough loci had been tested to insure their being scattered at varying genetical distances from the spindle fiber, and all factors had shown a frequency of homozygosis approximating 25 per cent, then the hypothesis that crossing-over occurs only between diagonals, and that the first division may be reductional or equational with equal chance would be well substantiated. However, with only five loci tested, alternative hypotheses may be proposed to fit the data if assumptions are made concerning the distance of the five loci from the spindle fiber. If crossing-over is as frequent between lateral homologous strands as between diagonal ones, the frequency of homozygosis of a factor would be 25 per cent with the first division always reductional at the spindle fiber if the locus of the gene were at such a distance from the attachment point that approximately half the tetrads would contain one crossing-over between the two points (multiple crossing-over in some of the tetrads would increase homozygosis); or again with the first division always equational, if the locus in question were at such a distance from the spindle fiber that all tetrads would contain one crossing-over between the two points (multiple crossing-over would decrease homozygosis). More definite conclusions must await the testing of other loci.

Analysis of Data for Linked Genes

Two series of crosses were made with linked genes. The first consisted of crosses of tapering females by males carrying the two recessives white and stumpy. The genes are situated in the sixth linkage group and show approximately ten per cent recombination. Ninety impaternate females were found in the F_2. A remutation to the recessive ivory, o^i (eyes), which is phenotypically white but is not linked to stumpy, occurred in the experiment. Impaternate females in which there was any doubt as to whether the eye color was due to white or ivory had to be discarded; sixty-four remained which gave data showing that the white eye color was due to the gene linked with stumpy. These were phenotypically 54 type, 2 white, and 8 white stumpy.

Attempts were made to breed all except those homozygous for both recessives. Those sufficiently tested to show their genotypes were as follows:

$$18\frac{+\ +}{wh\ st} : 12\frac{+\ +}{+\ +} : 8\frac{wh\ st}{wh\ st} : 3\frac{+\ +}{+\ st} : 2\frac{wh\ st}{wh\ +} : 1\frac{+\ +}{wh\ +}.$$

Such a tetrad as would produce the last class listed should also give rise to individuals homozygous for stumpy and heterozygous for white. The character *stumpy* fuses the tarsal segments into a mass at the distal end of the tibia so that stumpy females cannot hold on to the host caterpillars well enough to sting them. Even on caterpillars previously stung by other wasps they have difficulty in ovipositing so that they seldom produce large numbers of offspring. Two impaternate females with stumpy legs and black eyes were found and both produced a few progeny some of which had eyes phenotypically white. Unfortunately both occurred in fraternities where the remutation to ivory was segregating and counts of their progeny were not sufficiently large to test linkage with stumpy. No individuals of the type *wh st*/+ *st*, therefore, can be reported with certainty, although it is to be expected as an alternative to the + +/*wh* + type which was found.

The six individuals containing one non-cross-over strand and one cross-over strand prove that crossing-over occurs in the four-strand stage.

Three other possible types of females, *wh* +/*wh* +, + *st*, + *st*, and *wh* +/+ *st*, were not found. The first two would be produced only from tetrads in which there had been two occurrences of crossing-over in the region between white and stumpy. If crossing-over takes place only between diagonal strands, two cases of crossing-over between white and stumpy would also be necessary for the production of *wh* +/+ *st* females since it has been shown from the data on single loci that only laterals may pass to the poles together. If crossing-over between lateral homologues may occur as frequently as between diagonals, *wh* +/+ *st* females would result only from two occurrences of crossing-over between white and stumpy if the first division were always reductional at the spindle fiber, but they would constitute 25 per cent of all females from tetrads containing one crossing-over between white and stumpy if the first division were always equational at the spindle fiber, and 12.5 per cent if the first division were equational or reductional with equal frequency.

Numbers in this experiment were too limited to justify choice between the hypotheses. Further data involving two genes showing a higher percentage of recombination would give more individuals from tetrads containing one crossing-over between the marked loci and might afford critical evidence as to the ratio of the type analogous to *wh* + + *st*.

Table VI

Genetic analysis of F_2 impaternate females from crosses of tapering females by males recessive for second chromosome factors.

No. of individuals	Analysis of strands	Cross-over regions	
		1st strand	2nd strand
1	*C L N D / c l n D*	—	3
1	*C L N D / C L N d*	—	3
1	*C L N D / c L N D*	—	1
1	*Cc·LL·NN·Dd*	1	3
		or —	1, 3
1	*Cc·LL·NN·d?*	1	3
		or —	1, 3
2	*C L N D / C l n D*	—	1, 3
2	*c l N ? / c l ? ?*	2	?
1	*C?·L?·nn·??*	2	?
1	*c l n ? / c l n ?*	?	?
6	*C?·L?·N?·??*	?	?
1	*C L N D / c l n d*	—	—
1	*C L N D / C L N D*	—	—

The second experiment with linked genes involved crosses of tapering females with males carrying factors of the second linkage group: defective, *d* (venation); cantaloup, *c* (eyes); long, *l* (antennæ and wings); narrow, *n* (wings). The tapering females are homozygous for Minnesota-yellow, which also belongs to the second linkage group. Only 19 impaternate females were found in the F_2. In addition to the small number of progeny obtained, the series of factors proved unfortunate in several ways. Minnesota-yellow varies in the intensity of its expression and overlaps with type so that counts for this character are never wholly reliable and its position on the chromosome has not been satisfactorily determined. In this experiment results for Minnesota-yellow seemed too uncertain to add anything and it was finally completely disregarded. Cantaloup, long, and narrow are phenotypically good characters, easily recognized and showing no overlaps with type. They occur on the chromosome in the order named. Long shows about 11 per cent recombination with cantaloup, and narrow about two per cent recombination with long. Possibilities for breeding tests are limited, however, since homozygous narrow females are sterile, and homozygous long females have reduced fertility and must be provided with caterpillars previously stung. The character defective is controlled by one second linkage group factor, which overlaps with type, and several modifying factors. Without breeding tests it is impossible to say what is the constitution of any female, at the locus of the main factor for defective. Numerous linkage tests with defective, made by various workers, indicate that its location on the chromosome is beyond narrow on the same side of cantaloup. Recombination values vary widely from different experiments, approximating 20 per cent with cantaloup in one experiment and a slightly higher percentage with narrow in another (Whiting and Benkert, 1934).

Such analysis of the 19 impaternate females as it was possible to make is given in Table VI. Question marks are used where there were too few progeny to determine whether or not a recessive was present, and where counts of defective were uncertain. Wherever phenotypes or breeding tests showed how the factors present were arranged on the two second chromosome strands, diagonal lines are used to separate factors on one from those on the other. Where it was uncertain on which strand the factors occurred, the locus is set off by a period. The seven individuals listed first in the table must have resulted from four-strand crossing-over.

Possible Explanations of the Formation of Impaternate Females

The preceding material has been presented on the assumption that impaternate females are formed by the suppression of the second maturation division. If the second division takes place but the second polar nucleus reunites with the reduced egg nucleus, the same result would, of course, be produced.

Other possible hypotheses for the formation of impaternate females have been suggested by P. W. Whiting, and may be tested against the experimental data as follows. If both maturation divisions occurred, the diploid chromosome number might be reëstablished by splitting of the chromosomes, in which case impaternate females would always be homozygous for all loci; or any two of the four nuclei resulting from the maturation divisions might combine, in which case the frequency of homozygosis of any factor would be 16.6 per cent. Obviously the data will fit neither of these theories, homozygosis of the factors tested being too low for the one and too high for the other.

If both maturation divisions occurred and the embryo developed from union of one of the products of division of the first polar nucleus with the second polar nucleus (Richtungskopulationskern, suggested by Goldschmidt and Katsuki for *Bombyx*, 1928) or with the reduced egg nucleus, impaternate females would be heterozygous for all loci which had undergone reduction at the first division, and would have equal chance of being heterozygous or homozygous for loci which had undergone an equational first division. Frequencies of homozygosis approximating 25 per cent, which were found for factors at the five loci tested, would be obtained under this hypothesis if the first division of the tetrad were reductional at the spindle fiber, and at least one crossing-over occurred in every tetrad, and the locus under observation was so placed that the crossing-over took place between it and the point of attachment; or homozygosis would result in 25 per cent of the cases if the first division were equational at the spindle fiber and the locus were so placed that no crossing-over would have occurred between it and the point of attachment.

It is also possible to fit the data to various hypotheses involving derivatives from more than one oöcyte nucleus inclosed in the same egg.

IMPATERNATE GYNANDROMORPHS

Two gynandromorphs produced by virgin mothers were found in the F_2 generation from crosses of tapering or reverted tapering females to mutant males. The first was produced by a female heterozygous for tapering and white; it had a female abdomen, female ocelli, one female antenna with 14 segments, and one male antenna with 21 segments. The second was produced by a female heterozygous for crescent and had a female abdomen and a male head. Neither showed the recessives for which the mother was heterozygous, and since it was impossible to breed them the chromosome constitution could not be analyzed.

Linkage tests in *Habrobracon* are regularly made by counting the male offspring from virgin F_1 females so that there has been ample opportunity for observing impaternate gynandromorphs if they occur. However, these are the only two cases that have ever been found. Mosaic males are produced not infrequently from unfertilized eggs. Their phenotypes show that they have developed from two nuclei formed by the maturation divisions; they are males from binucleate eggs (Whiting, 1924, 1932*b*). A few mosaic males have shown such recombination of genetic characters as to prove that they originate from three haploid nuclei (oötids) derived from one oöcyte (Whiting, 1934*a*). It seems likely that the two impaternate gynandromorphs correspond with trinucleate males from other types of females. Since they are produced by virgins with the thelytokous tendency, the second maturation division may have been suppressed, resulting in a diploid nucleus that gives rise to female regions of the gynandromorph, while one oötid gives rise to the male regions.

Summary

1. Impaternate females, produced by virgin mothers, constitute on the average .702 per cent of the F_2 from outcrosses of tapering and reverted tapering females.

2. The constitution of the parental male, as well as the female, affects the ratio of F_2 females.

3. When F_1 virgins from outcrosses of tapering or reverted tapering females were X-rayed, the percentage of F_2 females fell to .164.

4. When tapering and reverted tapering females were inbred, the percentage of impaternate females in the F_2 was only .006.

5. The ratio of impaternate daughters decreases with increasing age of the mothers.

6. No impaternate females occurred in the F_2 from tapering males by type females, from shot-veins or Minnesota-yellow females by various mutant males, or from impaternate females by their brothers.

7. Enough impaternate females were produced by virgins heterozygous for one or more of five different recessive factors to indicate how frequently those loci undergo reductional or equational first divisions in the formation of the secondary oöcytes from which the impaternate females are assumed to arise.

8. For four of the five loci best tested the percentage of females homozygous for the recessive factor did not differ significantly from that of females homozygous for the dominant allelomorph, nor did either of these differ significantly from 25 per cent of the total. These results would be expected if crossing-over were always between diagonal strands and equational and reductional divisions occurred with equal frequency for any locus.

9. For the fifth locus, orange, homozygosis for the recessive was 16.5 per cent $(25 - 8.5)$; homozygosis for the dominant allelomorph was 34.3 per cent $(25 + 9.3)$.

10. The fact that homozygosis was well over 16.6 per cent except in the case of the orange recessive indicates that random assortment of the strands of a tetrad does not take place.

11. Impaternate females in the F_2 from tapering females by males carrying linked factors show that crossing-over occurs in the four-strand stage.

12. Two gynandromorphs were obtained from virgin mothers. Presumably they involved three haploid sets of chromosomes, two in one nucleus giving rise to female regions and a third in another nucleus forming male regions.

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SHELL REPAIR BY THE SNAIL PLEURODONTE ROSTRATA PFR.

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By his important study of the shell of snails, Levetzow (1932) has added to the evidence that some of his predecessors recorded, to show that there are differences between repairs as effected at the edge of the shell and elsewhere. Such differences he correlated with special functions of the mantle border and thus distinguished as "typical" and "atypical" growth. Since previous experiments have been chiefly upon snails of Europe, the objects of the observations summarized here were to find whether differences such as those described by Levetzow held good for the tropical land snails of the West Indies and also to determine the mode of joining new shell-formations to old shell.

The snails were brought to Baltimore, Md., from Cuba and bred, so that various ages were available. Pieces of the shells were removed, by breaking with forceps, by sawing, or by grinding on a wheel; and the processes of repairing were followed through various stages.

In immature shells, when a piece was removed from the thin edge of the aperture, the mantle border reached back locally and filled the breach with perfect shell having cuticle, ostracum, and hypostracum, to take the place of what was removed; and only then did the mantle border resume activity to advance the whole edge. The new shell, Fig. 2, shows continuation of the color band, but always a faint scar line where the old and the new join, caused by the failure of the new to quite come up to the level of the old. Sections show also that internally there is a confusion of structure where the new material is applied to the old. The case seen in Fig. 13 shows the old layers abruptly ending and new ones beginning after a short period in which the shell material was not normally organized. The color band is here represented by the dotted area ending abruptly in the old, and faintly starting in the new. In this section the drop in level was unusually great. The photograph (Fig. 2) shows four months repair after removal of part of the under surface and the outer edge of the shell. There resulted a permanent notch on the edge of the shell since the mantle border did not at first form the normal angle.

When, however, pieces were removed, not from the aperture edge, but from the apex (Fig. 5), from one or more whorls (Figs. 1 and 3), from the outer edge (Fig. 7), or from close to the aperture (Figs. 9 and 10), repair was made by a superficial layer of spherulites beneath which normal lower layers were then added. Such new parts have no color bands, no cuticle, no ostracum.

When the piece was taken out of the shell far from the aperture, the entire area of mantle exposed to the air secreted spherulitic material over its surface to bridge the gap in the shell. Also, in some cases the mantle shrank away from the shell around the exposure and then secreted in under the shell. In an extreme case the spherulitic layer ran in under the old shell 30 mm. before it rose up to solder to the old shell, but usually, as in Figs. 11 and 12, this layer fused to the shell near its broken edge, and was then underlaid by shell layers to complete the inner patch. These two sectional views are enlarged but fifty diameters and the spherulitic layers merely suggested; in reality some of the spherulites have a diameter of 60 $m\mu$ and some of their radii extend down into the lower layers. Figure 11 represents a cross-section of a kerf such as shown in Fig. 1. Figure 12 represents the edge of such repair as shown in Figs. 3, 5, 7.

When removal was close to the aperture, the mantle laid down the spherulitic layer progressively toward the aperture as shown in Fig. 9. Figures 9 and 10 show repairs on lower and upper surfaces near the aperture edge of *Pleurodonte* (*coracolus*) *arangiana* Poey. In adolescent snails that were making the peristome, as in Fig. 9, the mantle border no longer made outer layers even when the injury was at the edge of the aperture but made only spherulitic and lower shell layers.

At maturity, removal of part of the peristome led to secretion of peristome material in proper form and place to fill the gap (Figs. 4, 6, 8), but even after four months this repair lacked normal thickness, as seen in Fig. 6 in which a saw-cut is partly filled from the inside out. Sections of such repair work reveal that the mantle had forced the secreted material into the kerf in curved concentric layers.

Sections reveal the peristome as only inner layers rolled back over the ends of the cuticle and ostracum with concentric circles cut by long radii that represent such shorter lamellæ of other regions of the shell, as shown in Figs. 11, 12, 13.

When shell removal concerns several whorls, as in Figs. 1 and 3, the mantle of each whorl acts to make its own cover and a series of independent patches thus results; it is only the mantle border which can fuse whorls together under a continuous common cover. It is to be empha-

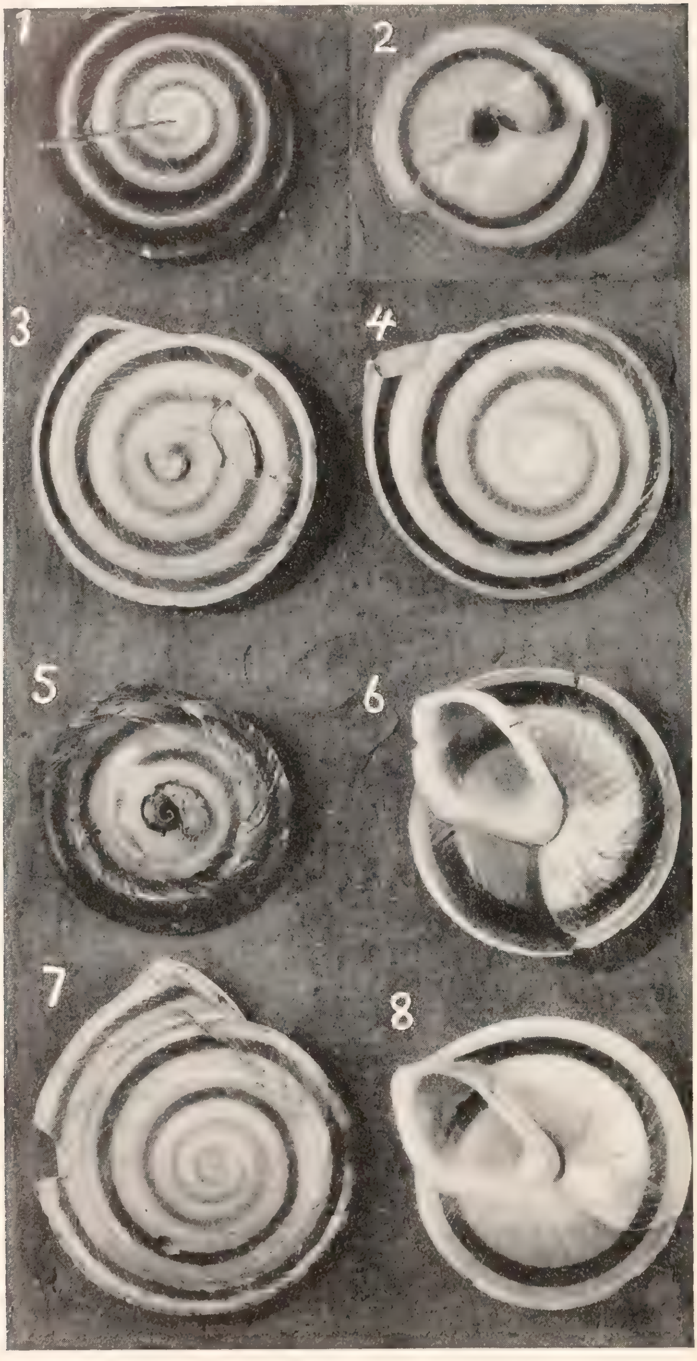
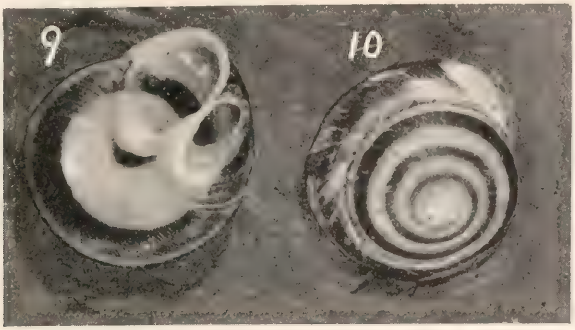


Plate I

sized that shell repair is the result not only of secretory, but also of muscular activities of the mantle. In immature shells the mantle border can inhibit its general activities, when a local part turns back to reform a lost shell area. The form and movements of the mantle border give shape to the shell and at maturity a change in secretions and in posture leads to the moulding of the peristome. The specific ability to make cuticle and ostracum is given up at maturity when the mantle border makes only inner layers. On the other hand, the general mantle surface makes only inner layers until as a reaction to exposure it produces a secretion of material crystallizing as spherulites.



Figs. 9–10 show stages in repair of holes made near the aperture.

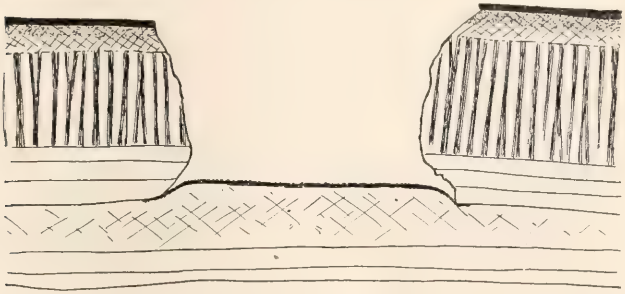


Fig. 11. A saw kerf, such as seen in Fig. 1, has been repaired by addition of new material across the breach and beneath the old shell seen to right and to left. The spherulitic layer against the air is represented as a dark line.

Plate I

All illustrations represent *Pleurodonte rostrata,* except Figs. 9 and 10, which are of *Pleurodonte arangiana.* Photographs are life size: but the three camera lucida sketches of ground sections are enlarged fifty diameters.

Figs. 1–8 show repairs after removal of various parts of the shells.

The general mantle surface has some slight form-giving function in case of removal of shell parts, since it may then either contract or expand and thus form either a shrunken or a protuberant substitute for the normal shell originally made by the mantle border.

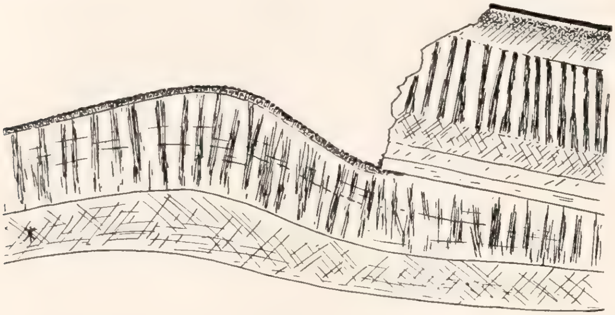


Fig. 12. Part of the edge of a hole in the shell repaired by new material continued in under the old shell. The new material is covered by a layer of spherulites where not soldered to the under face of the old shell.

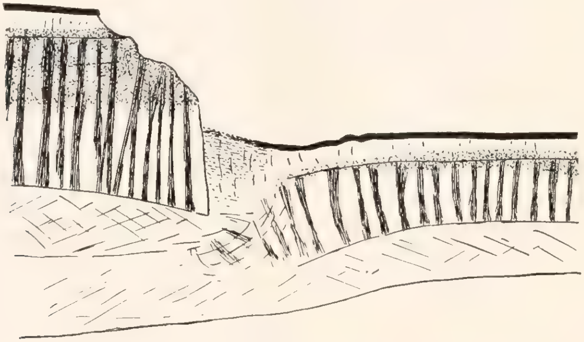


Fig. 13. The old shell above was the broken edge of the lip. The new shell lies on a lower level and extends under the old. Each has complete normal layers. There is no spherulitic layer. The junction lacks normal organization.

To conclude: as the methods of repair of the shell of a Cuban land snail taken at random agree with those of certain land, fresh-water, and marine snails of Europe, we may generalize that the methods of shell repair in gastropods may be either by "atypical" growth, where no cuticle is formed, and lime secreted as a sort of cement crystallizes as a covering of spherulites beneath which lower shell layers may be added; or by "typical" growth where a cuticle is secreted and repair is by repetition of former growth in which lime secreted beneath the cuticle takes on the complex organization of normal shell. But in the latter case there is, at least in this Cuban snail, a region where new joins to old in which perfect shell organization may be lacking.

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THE EGG-LAYING ACTIVITIES OF THE SEA HARE, TETHYS CALIFORNICUS (COOPER)

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The lavish way in which certain marine invertebrates spend sexual products to insure perpetuation of their species has long been a source of great interest to biologists. Early estimates were usually based on single spawnings without knowledge of seasonal production, and in many instances fell far short of the actual number of eggs laid in a season. Galtsoff (1930) has shown that the number of eggs laid by a Virginia oyster in a season may approximate one-half billion, which greatly exceeds 60 million, the highest previous figure.

The following account of the egg-laying of the sea hare, *Tethys californicus* (Cooper), in which the computations are correct to ± 5 per cent, adds more information as to what actually may be accomplished in one season by a marine animal. Table I gives a summary of the egg-laying activity of a sea hare weighing 5 pounds, 12 ounces, which laid 478 million eggs between October 29, 1933 and March 6, 1934, inclusive, or within four months and one week.

After each laying (27 in all) the eggs were drained of surplus moisture and then weighed. By measuring and weighing lengths of string from several layings it was found that one gram of egg string averaged 21.55 cm. in length. By multiplying the weight of the mass laid by 21.55, the total length of the egg string was obtained. In the 27 layings the sea hare laid a total of 60,565 cm. of egg string, 17,520 cm. of which were laid in the first laying. Also after each laying ten or more samples of the egg string were taken at different places throughout its length. From these samples the average number of capsules per centimeter was counted, the average for the entire lot being thirty-nine. The number of capsules per centimeter showed less variation than any other factor, the number of eggs contained in a capsule having no effect on its size. In fact, sometimes at the end of a laying several centimeters of string would be laid which would contain no eggs, or perhaps only a few, yet even in this portion of the string there would be about 39 capsules per centimeter. The number of eggs was counted from several capsules in each of the representative samples and the average computed.

These averages are given in column three of Table I. The average number of capsules per centimeter times the average number of eggs per capsule gave the number of eggs per centimeter. By multiplying the average number of eggs per centimeter in each laying by the length in centimeters, the total number of eggs for that laying was obtained, as given in column four of Table I.

TABLE I

Date of laying	Weight of eggs *grams*	Average no. eggs per capsule	Total number of eggs
Oct. 29	813	217	148,000,000
Nov. 5	135	190	21,587,000
Nov. 11	158	181	24,035,000
Nov. 15	76	166	10,550,000
Nov. 21	152	237	30,296,000
Nov. 25	97	180	14,674,000
Nov. 29	93	223	17,374,000
Dec. 5	96	227	18,277,000
Dec. 10	82	229	15,705,000
Dec. 18	85	230	16,450,000
Dec. 24	88	210	15,504,000
Dec. 29	58	192	9,392,000
Jan. 3	59	189	9,340,000
Jan. 10	92	215	16,709,000
Jan. 16	93	234	18,329,000
Jan. 19*	66	184	10,246,000
Jan. 22	53	179	7,988,000
Jan. 29	75	198	12,521,000
Feb. 1	74	191	11,831,000
Feb. 4	61	175	8,898,000
Feb. 8	60	185	9,288,000
Feb. 12	69	176	10,183,000
Feb. 17	46	163	6,235,000
Feb. 19	17	144	2,079,000
Feb. 22	32	132	3,583,000
Mar. 5	74	139	8,650,000
Mar. 6	6	87	466,000
Totals	2810	188 (av.)	478,190,000

* From this time on the eggs were infertile.

Sea hares are hermaphroditic and exchange sperm by copulation shortly before egg-laying. They segregate when mating, and from two to fifteen have been observed copulating in chains, all but the first and last giving and receiving sperm. In one instance eight were seen copulating in a ring, all giving and receiving sperm.

Eggs are laid in gelatinous strings 2.2 mm. in diameter, and the entire egg mass, which is usually cemented to eel grass or seaweed, resembles nothing so much as a tangled bunch of yellow yarn. Within the string the eggs are segregated in capsules and these capsules are spirally arranged along the string, four capsules to each spiral turn.

While laying, the sea hare takes the issuing string of eggs in a fold of the upper lip, and moving the head back and forth with no regularity as to distance, sticks the string together here and there. As the egg string passes from the lips it is covered with a sticky mucus and wherever the animal presses the issuing string against the mass it adheres to the portion already laid. This results in the apparent tangled mass. Within five or ten minutes after laying a mass of eggs, the animal resumes feeding and will continue until apparently it has replenished its fuel supply for another laying.

When one considers the complicated way in which the eggs are arranged in the string, the rate at which they can be laid is astounding. The sea hare lays its string of eggs at the average rate of 5.9 cm., or 230 capsules, or 41,000 eggs per minute.

The conditions under which the above layings took place could not have been optimum. The sea hare was kept with various other animals in an aquarium 5 feet long, 3 feet wide, and 2.5 feet deep. Ocean water was supplied to the aquarium at the rate of about 400 gallons every 24 hours from a gravity tank which was filled daily from the bay. The seaweed fed the animal was obtained from that which floated in with the tide; hence, feeding was irregular. The aquarium received no direct sunlight, it was crowded with other animals, and there was less aeration than under natural conditions. The supply of sperm ran out at the fifteenth laying, and from then on the eggs were infertile. The sea hare died two days after the last laying. It seems not unfair, therefore, to assume that under normal conditions the number of eggs laid would have exceeded the number laid and counted under aquarium conditions.

An individual egg measures 77 microns in diameter. Polar bodies are given off after seven hours, and the first division occurs at eleven hours. Practically all of the eggs become veliger larvæ which escape after twelve days.

You no doubt wonder what becomes of the millions of eggs and larvæ. Starfishes, if hungry, will eat the egg masses; and many larvæ are used as food by plankton feeders; but the greatest mortality occurs at and just after the time of settling. In the first place, since *Tethys* is limited to a diet of seaweed, only those larvæ which settle alongshore could be expected to survive. Secondly, those which settle alongshore have small chance of landing where they will be safe from being eaten by other alongshore animals. As the larvæ settle from the plankton, the small coiled shell measures about a half millimeter in its greatest dimension. Those that escape landing within the feeding range of the feet of barnacles, the tentacles of anemones and hydroids, the feeding gills of tube worms, or the siphons of the larger bivalves, are eagerly searched for by the lesser predacious animals, such as flatworms, nemerteans, annelids, and such Crustacea as small crabs, hermit crabs, and isopods, and by small fish, particularly gobies and cottoids (MacGinitie, 1934). After *Tethys* larvæ fully metamorphose and reach a size of three or four millimeters they seem to become distasteful to other animals, and in this respect may be classed with the nudibranchs in general. The time of settling is perhaps the time of highest mortality for all alongshore or mud-dwelling marine animals which have free-swimming larvæ.

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THE EFFECTS OF X-RADIATION ON THE RECONSTITUTION OF STENOSTOMUM TENUICAUDA AND SOME OTHER WORMS

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Several papers (e.g., Curtis and Hickman, 1926; Stone, 1932; Butler, 1933; Gianferrari, 1929; Turner, 1933), have reported the use of X-radiation as a means of studying reconstitution and regeneration in those forms exhibiting these phenomena. If parts of the organism are removed following exposure to dosages of X-rays, the reconstitutional processes that normally lead to replacement of the missing parts are either retarded or stopped entirely, depending on the dosage used. This result has been attributed by Curtis to the selective action of the X-radiation upon certain cells of the organism essential to reconstitution. The present paper reports an attempt to influence the reconstitutional behavior of a rhabdocœl, *Stenostomum tenuicauda,* by means of X-radiation and, for comparative purposes, the effects of some similar dosages on two more highly organized worms, *Dero limosa* and *Pristina longiseta.*

Stenostomum tenuicauda, a minute rhabdocœl turbellarian commonly found in fresh-water ponds, ordinarily consists of a chain of zoöids separated by fission planes. Two types of head reconstitution are present in this species: (1) transverse sections of the worm containing one or more fission planes in the absence of the head of the chain show resorption of anterior partial zoöids and of younger zoöids by an older posterior head region which becomes the head of the reconstituted worm; and (2) a headless individual zoöid, containing no fission plane at the time of section, reconstitutes a completely new head at the anterior end of the piece (Van Cleave, 1929). Only the latter type, in which the structures of the new head are formed *de novo,* concerns us here.

Worms consisting of two zoöids separated by an immature fission plane were selected for testing reconstitutional behavior following irradiation. From each zoöid of such a worm could be obtained a piece, freed of preformed head-structures by transverse cuts, which in control worms reconstituted new heads in about 90 per cent of the cases. Failure of new heads to form can usually be ascribed to improperly placed cuts which leave the piece too short. The usual length of the whole worm was 1.5–2.0 mm.; both the anterior and posterior headless pieces comprised one-half or more of its original zoöid. Each zoöid sectioned for reconstitutional capacity was isolated and record kept of its size, its position in the parent worm, the amount removed and, from day to day, the evidence of reconstitution. All the work was done under the binocular dissecting microscope.

Two-zoöid worms were preferred throughout the experiments; a cut through the base of the pharynx removed the head and all anterior parts and two more cuts removed the fission plane and head region of the posterior zoöid. Since the tail was left intact, the anterior piece presented two cut surfaces, the posterior only one. The cut surfaces contract at once to close the wound; the first evidences of new head formation usually are seen within 24 hours and appear as a small depression on either side of the cut anterior end. These are the ciliated pits, connected with the cerebral ganglia, and the head rapidly takes morphological form after their appearance. Within two or three days after sectioning, the piece has entirely reconstituted itself, having formed a new head, mouth, pharynx, and tail where the tail has been removed. In many cases after reconstitution has been accomplished a fission plane arises and a second zoöid appears; presumably the actual growth of the worm, if it occurs, is based on foodstuffs present in the gut at the time of sectioning, since all cut pieces were kept in boiled and filtered tap-water.

The irradiation was done with equipment of the mechanically rectifying type, energizing a broad-focus Coolidge tube. The Rœntgen machine was calibrated with a Wulf ionometer, the latter in turn calibrated in Germany with the proposed R unit. The operating factors, unless otherwise stated, were as follows:

127 kilovolts (peak).
5 milliamperes.
30 centimeters, target distance.
No filters.

At this distance one minute of unfiltered radiation comprised approximately 140 R units of intensity.[1]

In some experiments the exposure time was reduced by halving the target distance; the heat factor introduced by this procedure was minimized by the use of ice-packs around the dishes containing the worms and by an electric fan. Similarly cooled control worms showed no ill effects nor did the irradiated worms with such protective treatment react as did worms when the temperature of their surrounding medium was deliberately raised. After experimenting with the use of individual isolation slides and with boxes of heavy lead, it was thought best to irradiate all worms in mass cultures in Syracuse dishes 2.5 cm. in diameter which contained about one cubic centimeter of water and which could be covered with a round cover slip. The effects of secondary and scattered radiation were disregarded.

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[1] I am indebted to Dr. J. L. Weatherwax, physicist of the Philadelphia General Hospital, for his kindness in calibrating the Rœntgen machine.

Immediately after irradiation the worms were washed by transferring them through several changes of boiled and filtered tap-water and some sectioned at once with the purpose of studying the immediate effect of the irradiation upon their reconstitutional capacity. Other worms were individually isolated in Syracuse dishes, in order to follow from day to day any gross effect of the irradiation that might appear in the unoperated worms. The remainder of the irradiated set of worms was kept as a mass culture from which worms were taken after varying intervals of 2–14 days to be sectioned with the purpose of studying the possibly delayed effect of irradiation upon their reconstitutional capacity. The days intervening between irradiation and sectioning constituted a period of latency in which such an effect might develop.

For comparison with the effects of irradiation on *Stenostomum,* two species of microdrilous oligochætes, *Pristina longiseta* and *Dero limosa,* were also irradiated and later sectioned for reconstitutional capacity. These worms were, like *Stenostomum,* grown in laboratory cultures.

EXPERIMENTAL

Preliminary exposures of the worms to filtered radiation made it evident that if any gross disturbances in either structure or reconstitution were to be detected, the doses must be of a high order of magnitude. With the apparatus used, 180 milliampere-minutes of exposure with a filter of 6 mm. aluminum gave approximately 800 R units. One thousand R units left the worms apparently unaffected, as shown by mass and isolated cultures and by normal reconstitutional behavior. One set of 20 *S. tenuicauda* received a dose of 2,000 R units, had their heads removed immediately after irradiation, and reconstituted normally in 48 hours. Four days after the initial irradiation the same reconstituted worms received a dosage of 1,000 R and were sectioned and again reconstituted new heads in 48 hours. Eight days later the set was apparently normal and unaffected. To obtain greater intensity, the use of filters was hereafter discontinued.

Successive dosages were next tried in the quest of a lethal dose. A dosage of 1,680 R units, applied daily for six consecutive days to massed groups of *Stenostomum, Pristina,* and *Dero,* left the rhabdocœls undisturbed. *Dero* likewise was unaffected—both head and gill regions regenerating by 4 days and the cultures of irradiated worms thriving 30 days after the raying. Irradiated specimens of *Pristina*, however, gave evidence of abnormality in two slowly and incompletely reconstituted heads among an otherwise normal set of 20. Because of the daily handling involved, successive dosages were abandoned to try the effect of a single, continuous dose of great magnitude.

Mass cultures of *Stenostomum, Pristina,* and *Dero* were exposed to a dosage of approximately 20,160 R units. The operating factors were as follows:

127 P. K. V.
5 Ma.
30 cm. target distance.
144 min. exposure.
No filters.

The dosage was applied over a period of 4½ hours; the tube was allowed to cool 5 minutes and 30 minutes alternately at the end of 18-minute periods. A thermometer placed next the dishes containing the worms registered no increase in temperature from heat radiation from the tube. All the worms seemed normal in appearance and behavior at the end of the irradiation. The subsequent procedure will be described separately for each species.

Pristina longiseta

No difference between the irradiated and control sets was noticeable during the first two days after irradiating; at three days, however, the worms of the former set were surrounded by mucus, with consequent clumping, but were apparently structurally sound. Later the mucus disappeared and during the remainder of the 26 days the worms were kept under observation the irradiated worms were individually indistinguishable from normal control worms. There were noticeably more worms in the control set although fission planes were not absent in the irradiated stock. Since no signs of cytolysis or disintegration were observed in the culture during this period the disparity in numbers was attributed to a relative decrease in formation of new fission planes in the irradiated worms.

For the effect of the irradiation on regeneration the five head segments were removed from a group of treated and untreated worms. The head in *Pristina* is made up of the five anterior segments, each marked by a pair of bristles, plus the proboscis. Following the loss of from one to five of these anterior segments the worm regenerates only the loss. If six or more anterior segments are removed, only five are regenerated (Hempelmann, 1923). Five days after the removal of the anterior segments the irradiated worms presented a typical picture in which a short conical outgrowth, free of bristles and setæ, appeared at the cut anterior surface. By the same time the cut untreated worms had regenerated a new head of five segments, complete with bristles, setæ, and proboscis. Where a fission plane was present in the irradiated worm, separation occurred and the anterior piece died in about a week, showing no further change at the anterior end.

Dero limosa

For the first five days after irradiation *Dero* seemed unaffected, but on the sixth day the anterior part of the intestine was often found to be opaquely black and both posterior and anterior ends of the worm were swollen with large blister-like structures on the surface. Cytolysis set in shortly, producing many short headless pieces. The black metallic color persisted in the remaining long worms which were clumped together in contrast to the scattering of worms in the untreated culture. These latter were numerous and large, with fission planes. All the irradiated worms eventually disintegrated, none living more than 18 days. The controls remained in excellent condition. Pieces cut for regeneration cytolyzed before definite results could be obtained.

S. tenuicauda

The mass cultures of irradiated worms were kept under observation for 26 days, during which period no abnormalities were noted. At no time was there any observable difference between the irradiated worms and untreated control cultures. After remaining without food for three weeks both cultures were fed and fission planes soon appeared in great profusion.

Worms were sectioned, with the purpose of studying reconstitutional capacity, immediately after irradiation, and also, in order to allow periods of latency, 3, 5, 6, and 14 days after irradiation. The worms used were the two-zoöid type with only one fission plane; the anterior head was removed by a cut across the pharynx and care was taken that all the head region of the posterior zoöid was removed. The reconstitutional results are shown in Table I.

The effects of allowing a period of latency between irradiation and cutting were clear with this dosage. Those worms cut within the hour following irradiation closely approximated the reconstitutional behavior of control worms and although at the end of nine days a few more ir-

TABLE I

Reconstitution of headless zoöids of Stenostomum tenuicauda following an irradiation of approximately 20,160 R units

Days after cutting	1				2				3				4				6				9			
	M	H	C	N	M	H	C	N	M	H	C	N	M	H	C	N	M	H	C	N	M	H	C	N
Cut at once	0	18	2	0	3	4	11	2					20	0	0	0								
20 xx	1	16	3	0	1	2	13	4	1	0	7	12	1	0	0	19					8	0	0	12
20 cc	1	18	0	1	1	4	14	1	1	1	3	15									4	1	0	15
3 days latency																								
20 xx	9	10	1	0	9	3	7	1	9	0	3	8					9	0	0	11	11	0	0	9
10 xx	5	5	0	0	5	2	3	0	5	2	1	2	5	2	1	2	6	1	1	2	9	0	0	1
10 cc	1	9	0	0	1	0	8	1	1	0	0	9												
5 days latency																								
13 xx	7	5	1	0	7	5	0	1	7	5	0	1					7	5	0	1	11	0	0	2
6 cc	0	6	0	0	0	6	0	0	0	0	6	0												
6 days latency																								
20 xx					10	4	6	0	10	1	4	5					11	0	0	9				
14 days latency																								
10 xx	0	10	0	0					0	0	0	10												

M = missing or dead.
H = headless.
C = with ciliated pits.
N = normal, i.e., completely reconstituted.

xx = irradiated worms.
cc = control worms.

radiated worms were missing than in the controls, the observations made on the fourth day show plainly that the missing worms had reconstituted new heads before their death. However, when several days were allowed to lapse between irradiation and cutting, a difference between control and irradiated worms was apparent. Approximately half the irradiated worms in each set died during the 24 hours following section. Disintegration began about four or five hours after cutting and nothing remained of the piece on the following day. The greatest mortality occurred in worms cut four and five days after irradiation. Of those surviving the cutting, either the head was reconstituted or the headless piece disintegrated, none of the latter surviving more than a week.

This susceptibility of apparently normal irradiated worms to cutting, following a period of latency, lasted for at least six days but eventually disappeared, for irradiated worms cut 14 days after irradiation showed no sign of it. This might be due to the death of those worms which were affected by the radiation, the ones surviving and cut being unaffected. Individual isolation of irradiated worms was not made with the sets shown in Table I, but with subsequent dosages of the same intensity this procedure was carefully followed and deaths of isolated worms within periods corresponding with the above latencies were never observed. Moreover, mortalities of 50 per cent would presumably be apparent even in mass cultures yet no difference in numbers of worms in mass cultures of the above sets and controls was observed.

A comparison of the differential mortality of cut pieces of anterior and posterior zoöids of the same worm indicates that the explanation for the mortality of worms cut following latency lies in the differential susceptibility of their anterior and posterior zoöids. The fate of the paired pieces of the same worm in a representative group of 34 two-zoöid worms can be summarized as follows:

Both anterior and posterior pieces normal	8
Both anterior and posterior pieces missing	10
Anterior piece normal—posterior missing	3
Anterior piece missing—posterior normal	13

In a genetic study of *S. incaudatum,* Sonneborn (1930) found a real difference to exist between lines derived from anterior and posterior zoöids as indicated by rate of reproduction and susceptibility to lead acetate. Posterior individuals were "potentially immortal"; anteriors weakened and died as well as being more susceptible to the toxic agent. It is suggested that *S. tenuicauda* likewise exhibits this differential between anterior and posterior zoöids as shown by the greater susceptibility of anterior pieces to disturbances introduced by sectioning after a period of latency following irradiation. No data are available as to the possible anterior origin of individuals in which both anterior and posterior piece failed to survive.

A larger dosage, of approximately 30,240 R units, was given the three species of worms under conditions identical with those last described. The irradiation was applied in 18-minute exposures over a period of nine hours. The mass culture of irradiated *Dero* showed cytolysis and blackening of the gut within 48 hours and all the worms were completely disintegrated by seven days. Neither *Pristina* nor *Stenostomum* gave any evidence of abnormality during the 11 days they were kept under observation. Of 20 *Stenostomum* cut after 4 days latency, 15 had reconstituted new heads by 72 hours while 2 were missing and 3 still headless. Two days later one of these was missing while the other two were normal worms. This enormous amount of irradiation had no effect on the reconstitutional capacity of *Stenostomum*, irrespective of the possibility of its lethal effect at some later date.

A detectable effect on the reconstitutional behavior of *Stenostomum* might be obtained if the irradiation could be concentrated in point of time. Since the apparatus was being run at capacity, such concentration could only be obtained by appreciably decreasing the worm target distance. Accordingly, in order to quadruple the intensity, the dish containing the worms was moved 15 cm. from the target and provisions (described above) made to avoid temperature effects. Some results with a relatively low dosage of approximately 7,000 R units are shown in Table II. Reconstitution has proceeded normally and no clear effect from delay in cutting the pieces after irradiation is noticeable. When worms are missing three or more days after cutting, always presumably from disintegration, reconstitution has preceded the disappearance. Similar results were obtained with a dosage of 13,900 R units. Single intact *Stenostomum,* isolated immediately after irradiation, gave no indications of abnormality during the 16 days under observation. Of a set of 20 isolated worms, all had formed new fission planes upon being fed and, as a result of asexual division, increased in numbers.

The largest concentrated dosage given during this series was one of approximately 20,160 R units given *Stenostomum* in 36 minutes. Both experimental and control worms were kept at a temperature of 50° C. for 20 hours preceding the exposure and the ice surrounding the worm container was renewed during the irradiation, as was necessary because of the heat radiated by the tube. Reconstitution occurred normally within 48 hours after irradiation in worms cut immediately following the raying. But all were completely disintegrated by 96 hours after irradiation, as were also the isolated whole worms. The control worms in each instance were entirely unaffected.

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TABLE II

Reconstitution of headless zoöids of Stenostomum tenuicauda following an irradiation of 7,000 R units. Abbreviations as in Table I.

Days after cutting	1				2				3				4				6				14			
	M	H	C	N	M	H	C	N	M	H	C	N	M	H	C	N	M	H	C	N	M	H	C	N
Cut at once																								
20 xx	1	18	1	0	1	2	16	1					1	0	0	19					4	0	0	16
20 xx	0	16	2	0	0	3	17	0					2	0	0	18								
20 cc	0	19	1	0	0	1	18	1	0	0	2	18	0	0	0	20	3	0	0	17	5	0	0	15
2 days latency																								
20 xx					2	0	5	13					2	0	0	18					8	0	0	12
20 xx	1	16	3	0	1	1	5	13					1	0	2	17					9	0	0	11
4 days latency																								
20 xx	4	16	0	0	4	1	9	6									8	0	0	12				
20 xx	3	16	1	0	3	2	10	5	5	0	0	15					9	0	0	11				

CONCLUSION

The manifest resistance of *Stenostomum* to huge doses of X-radiation is a phenomenon the counterpart of which is seen in other of the lower organisms, particularly the Protozoa. Crowther (1926), for example, had to give *Colpidium* the enormous dose of 80,000 R units in less than 20 minutes if he wished to kill the cells. *Stenostomum* survived 30,000 R units with the exposure extended over a period of 9 hours but succumbed to a concentration of 20,000 R units delivered in 36 minutes.

The lethal effect of the irradiation is subordinate, however, to the fact that normal reconstitutional processes are apparently uninfluenced by the irradiation. Because of the rapidity with which a partial headless zoöid is transformed into a morphologically complete worm, it is possible to observe complete reconstitution in irradiated worms which subsequently undergo complete cytolysis. The mortality among those worms in which a number of days has intervened between the irradiation and the cutting may represent a latent effect of the irradiation. But it would seem as if this effect lay more in the direction of inducing a lethal susceptibility to factors involved in cutting away parts of the zoöid rather than in the retardation of the normal processes of reconstitution, for instead of resulting in the usual headless pieces which persist after wound closure for as long as six days, these irradiated pieces start to disintegrate in four or five hours after cutting. In this sequence of events I see nothing comparable to the situation in irradiated *Pristina* where a short amorphous outgrowth following the removal of the head segments indicates the loss of anterior regeneration.

SUMMARY

1. Laboratory-grown specimens of *Stenostomum tenuicauda* were subjected to large dosages of unfiltered X-radiation and the effect upon their reconstitutional behavior following operation noted. For comparison, similar observations were made on *Dero limosa* and *Pristina longiseta*.

2. Maximum dosages of approximately 30,000 R units, applied over an interval of nine hours, and of approximately 20,000 R units applied in 36 minutes had no apparent effect on normal reconstitution in *Stenostomum*. The lethality of the latter dosage was indicated by the disintegration of the completely reconstituted worms and of isolated unoperated worms four days after the irradiation.

3. Anterior head regeneration in *Pristina* was inhibited following an apparently non-lethal dosage of 20,000 R units applied over a period

of four and a half hours. Specimens of *Dero* were cytolyzed by the same dosage.

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THE STERILIZATION OF PROTOZOA

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Introduction

The recent development of techniques for the culture of Protozoa in solutions containing known bacteria and in sterile solutions (pure culture), demands a method for the complete removal of the bacteria normally found in their environment.

Several methods have been used, namely, migration through tubes of sterile medium (Purdy and Butterfield, 1918; Glaser and Coria, 1930), migration over an agar surface (Oehler, 1919), chemical treatment of cysts (Luck and Sheets, 1931), irradiation (Taylor, et al., 1933), and washing in successive dishes of sterile water (Hargitt and Fray, 1917; Parpart, 1928). Of these the last is the most generally applicable and dependable.

As developed by Parpart for *Paramecium*, the technique of washing overcomes the difficulties caused by the presence of ordinary bacteria and spores which may be defecated in a viable condition, but it does not overcome the difficulties caused by the presence of extraordinary bacteria (Hetherington, 1933, 1934*a*, 1934*b*). Moreover, a rather elaborate hood was used by Parpart.

In the present paper a simple technique is described which overcomes these difficulties. It combines washing with migration, reduces the number of washings from 10 to 7, increases the efficiency so that a considerable number of animals can be sterilized simultaneously, and greatly simplifies the apparatus required.

Briefly, the technique consists in inducing the Protozoa to migrate for some distance in order to separate them from surrounding bacteria, then allowing them to swim about for a period to permit the shedding of attached bacteria.

Apparatus

The apparatus used for the experiments was as follows:

Dissecting binocular with 55-mm. objective and side rests for hands; 12 Columbia dishes;[1] 12 Petri dishes; 10-cc. graduated pipettes; large test tubes; transfer pipettes (medicine droppers) drawn out to internal capillary diameters 1 mm. and 200–300 μ; cotton and a wooden rack, i.e., a board about 6 cm. wide by 60 cm. long elevated to the level of binocular stage.

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[1] Watch glass No. 9851, Arthur H. Thomas Co., Philadelphia.

Sterilization of Apparatus

The Columbia dishes are enclosed in the Petri dishes which are then wrapped in paper. The graduated pipettes are cotton-stoppered at the larger end, and individually wrapped in paper. After removal of the rubber bulbs, the transfer pipettes are put into the large test tubes, which are cotton-stoppered. They are sterilized along with the dishes and pipettes in hot air, 160–170° C. for one hour. It is an advantage to work in a room in which the air is relatively quiet.

Medium

Bacto Yeast Extract	1 gram
Peters' Medium	500 cc.

Dispense into Pyrex tubes or flasks, cotton-stopper, and autoclave, 15 pounds for 5 minutes.

Preparation of Peters' Medium.—Put about 10 grams of $Ca(OH)_2$ (powdered Kahlbaum, pro analysi) into about a liter of doubly distilled water; cork and shake. Allow to stand for two days, shaking a few times in the interim. Pass CO_2 through this solution (at 23° C.), using either commercial tank CO_2 or a generator, for one hour. Cork and allow to stand for 24 hours. Pour off the slightly cloudy supernatant fluid into two 500-cc. good quality ground glass-stoppered bottles. Pass CO_2 through each for a minute, stopper, and label: $Ca(HCO_3)_2$, 0.1 M., 55 cc./l.

Prepare 0.015 M $MgSO_4$, K_2HPO_4, and 0.0075 M Na_2HPO_4 in doubly distilled water, and add " 10 cc./l." to the label of each. A liter of Peters' medium is prepared by diluting 55 cc. of the calcium bicarbonate and 10 cc. of each of the others to one liter. Do not shake into equilibrium with air.

Procedure

The organisms to be washed should be very vigorous and well nourished. If a large number in poor condition is available it is often possible to prepare them for washing as follows. Put a small mass of favorable bacteria removed from a nutrient agar plate into a dish of medium. Transfer 0.5 cc. of concentrated Protozoa to this dish, and leave 3 or 4 hours. Many kinds of bacteria are favorable for this purpose (Hetherington, 1934*a*); *Achromobacter inunctum* is probably as generally useful as any. The method of washing follows.

Arrange five of the Petri dishes enclosing Columbia dishes on the rack. With a 10-cc. pipette, put 1 cc. of sterile medium [2] into each, raising the Petri cover just enough to permit introduction from the side.

Wash 1, migration.—Place the first dish on the binocular stage. With the large bore transfer pipette, put a drop of concentrated Protozoa to be washed into the left margin of the first dish so the inoculum is as sharply demarked from the medium as possible. Some of the Protozoa will migrate to the opposite side in a few seconds. The cover of the Petri dish may be left off.

Wash 2, migration.—Transfer 15–25 of those which migrated to the right side, using a small bore pipette, to the left margin of the second dish.

Wash 3, 3 hours.—Transfer those which migrate across the dish to the third dish, without entirely removing the Petri cover, and leave them for three hours.

Wash 4, migration.—Remove the cover, transfer all the Protozoa to the left margin of the next dish and leave them until some have migrated to the opposite margin.

Wash 5, 3 hours.—Transfer those which migrate to the fifth dish and leave them three hours.

Wash 6 and 7, final migration.—Place the sixth and seventh dishes on the rack and fill with medium. Put all the organisms into the left margin of the sixth dish and proceed with two more migrations; from this dish introduce them directly into the culture medium. Complete migration to the opposite side of the dish is not essential. Care should be taken to keep the last Petri dish cover over the dish as much as possible.

Experimental Basis for the Method Described

Variety of bacteria which must be removed.—The empiric sterilization of a number of different Protozoa from a variety of sources brings out a point of basic importance. The characteristics of bacteria which infect Protozoa from different sources may be diverse in the extreme. The number of strains the investigator may need to remove in order to sterilize Protozoa derived from natural waters is practically infinite. This explains why Parpart found a delay of 5 hours necessary for the defecation of viable spores, while Glaser and Coria (1933) obtained hundreds of paramecia free from such bacteria after one migration through a tube of sterile medium.

[2] The manipulation of sterile solutions is carried out using the flame technique. It is much more effectively demonstrated by a good bacteriologist than described. The rigid practice of this technique is absolutely necessary for success.

A table showing the number of washings required to sterilize a particular species derived from one pond would therefore have a very limited value. However, sterilizations from cultures on a single known strain of bacteria (zweigliedrige Kultur) give reproducible results which throw light upon the way in which bacteria are displaced. Table I gives results with *Colpidium colpoda* grown on *Aerobacter aerogenes* in 0.2 per cent yeast extract-dextrose.

The entire content of each dish was put into a tube of nutrient broth in order to make the test for bacteria. The results presented in Table I demonstrate that were it not for adhering bacteria, the second wash would be sterile in each experiment, since the first involves migration through sterile medium. This is proved in the experiment with a single colpidium from a cloudy culture. Here the second wash was sterile, yet the organism was infected, all bacteria being finally eliminated in the fourth wash.

TABLE I

The sterilization of different numbers of *Colpidium* growing on *Aerobacter aerogenes.* "Cloudy," *Colpidium* from thriving culture containing great numbers of bacteria; "clear," from a similar culture in which a majority of bacteria had been eaten. *M*, migration; III, 3-hour bath; +, infected; −, sterile.

Initial number of colpidia		Washings						Final number of colpidia
		2	3	4	5	6	7	
		M	III	M	III	M	M	
Cloudy	25	+	+	+	+	+	−	15
	15	+	+	+	−	−	−	7
	1	−	+	+	−	−	−	1
Clear	170	+	+	+	+	+	−	100
	25	+	+	+	−	−	−	15
	1	−	−	−	−	−	−	1

That the bacteria actually do become wedged between the cilia is indicated by results obtained in the sterilization of *Euplotes patella,* a hypotrichous ciliate having no cilia (only cirri). Sterilization from cloudy cultures of *Achromobacter pinnatum* and small numbers of unknown bacteria was achieved with three migrations and no baths.

Sterilization of large numbers.—The number of adhering bacteria is roughly proportional to the number in the environment. This is demonstrated by the sterilization of colpidia which had caused their environment to appear clear to the unaided eye (Table I). These results were verified on other Protozoa, including *Paramecium caudatum*. To apply to the latter animal, the number of infected dishes (+) recorded in the table must be increased by one or two for each row. The success with 100 ciliates suggests a method for obtaining large numbers. Unfortunately this does not apply to Protozoa from clear natural waters, which may indeed harbor the most tenacious and deceiving bacteria.

Adherent bacteria.—Peters (1921) and Glaser and Coria (1933) described pure cultures of *Colpidium* and of *Paramecium*. Hetherington (1933, 1934*a*) presented evidence that the rod-like bodies which they described and interpreted as discarded cilia were actually slow-growing bacteria. These bacteria are apparently harmless, and permit the growth of ciliates after they have increased to sufficient numbers. When colpidia contaminated with this bacterium were put in a dilute solution of ammonium glycerophosphate, growth occurred only after a lag of 10 days. Microscopic examination of this medium revealed small adherent rods apparently quite similar to those which were mistaken by Peters for discarded cilia. When these contaminated ciliates were inoculated into a medium capable of supporting their growth in pure culture, 1 per cent yeast extract, they started to grow immediately and grew vigorously, but the bacteria remained. Attempts to remove them, using sterile tap-water as the washing fluid, failed. Replacing the sterile tap-water with yeast extract yielded sterile ciliates. The heightened activity of the bacteria and possibly also of the locomotor organs of the ciliates may explain the difference. In testing for them the culture should be subjected to careful microscopic examination. Plates of the medium should be incubated for a week at 25° C.

Another series of results shows that a complex medium is preferable to water as a washing medium. These have to do, first, with certain acid-forming bacteria of rather tenacious nature; second, with spore-forming bacteria. The first-named are not understood (Hetherington, 1934*b*), but the unfavorable reaction of contaminated cultures of *Colpidium colpoda* is very well defined. In some respects they resemble anærobes. It was found that when very vigorous animals were washed through two baths of three hours each, separated by a migration, as many as five could be obtained sterile.

Spore-forming bacteria.—Speaking in general, spore-forming bacteria are infrequent contaminants and when present are easily removed. *Bacillus niger* was more easily removed than *Aer. ærogenes* (Hetherington, 1934*b*). Probably culture in air-contaminated shallow vessels of 0.7 per cent hay, the source of Parpart's paramecia, enriched spore-formers. However, under certain conditions the removal of ingested spores is difficult, and these are related to the level of vitality of the Protozoa.

Importance of vigorous metabolism.—If the Protozoa are not very vigorous at the outset, and if a non-nutrient medium is used, no amount of washing can be depended upon to yield sterile animals. Microscopic examination of ciliates in this condition usually reveals a few food vacuoles in the posterior portion of the body where they remain until death or the resumption of vigorous metabolism.

Thus the speed with which retained bacteria and spores are discarded is profoundly affected by the physiological state of the organism at the time it is washed. Ciliates which actually multiply during the process (*Glaucoma pyriformis*) are very easily sterilized. On the other hand, larger forms appear to be increasingly less well adjusted to small volumes of medium in shallow dishes, and are inclined to retain spores and viable bacteria for long periods.

Of an extensive series of media tested, the yeast extract was found to maintain vigorous metabolism more uniformly than any other particle-free system. Ciliate Protozoa in particle-containing media frequently fail to migrate.

Finally, a nutrient washing medium is desirable because the culture medium into which they are to be finally inoculated is generally a nutrient medium, and all possible stresses of adjustment are to be eliminated during the wearing process of washing.

Difficulty in sterilization, a function of size.—The difficulty of sterilizing Protozoa varies roughly as the size. Thus *Colpidium colpoda,* twice as large as *C. campylum,* is uniformly more difficult to sterilize. *Paramecium caudatum* is always more difficult than *C. colpoda.* Other factors, of course, enter in to alter any precise relation. Thus *Chilomonas,* a small saprophytic cryptomonad, and *Euplotes* are more easily sterilized because of the lack of cilia, while *Glaucoma pyriformis,* about as large as *C. campylum,* is easier to sterilize because of the very vigorous growth engendered by the yeast extract medium. On the other hand, the large ciliates *Stentor* and *Bursaria* have never been obtained free of bacteria. The technique presented is dependable only for those Protozoa which can be grown in pure culture or in zweigliedrige Kultur.

Air contamination.—External contamination from the air during the washing process must be avoided. The presence of Petri dish covers over the wash dishes effectively precludes trouble from this source. The effect of leaving off the covers was tested. Protozoa were sterilized with all the dishes open to air. Success was achieved under these conditions only when the sterile medium was put into the last dish just before use, and migration consummated promptly.

Summary

1. A method of sterilization combining the technique of migration with that of washing is presented.

2. The method permits the sterilization of large numbers of Protozoa at once when the infecting bacteria are known and favorable.

3. It permits the sterilization of Protozoa which are contaminated with very adherent bacteria which cause particular difficulty.

The author wishes to thank Professor S. O. Mast for critical reading of this manuscript.

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PROGRAM AND ABSTRACTS OF SCIENTIFIC PAPERS PRESENTED AT THE MARINE BIOLOGICAL LABORATORY

July 3, 1934

OVARIAN HORMONES AND BASAL METABOLISM. Mary E. Collett.

THE CARBON DIOXIDE TENSION IN THE BRAIN. Laurence Irving and Mary Scott Welch.

A METHOD FOR THE ESTIMATION OF CHANGES IN THE RATE OF INTESTINAL SECRETION. E. S. Nasset.

TRANSFER OF NEUROHUMORS. G. H. Parker.

July 10, 1934

SOME PHENOMENA PRODUCED BY CENTRIFUGING SEA URCHIN EGGS BEFORE AND DURING CLEAVAGE. Ethel Browne Harvey.

WATER CHANGES IN TROUT EGGS AT THE TIME OF LAYING. Jeanne F. Manery and Laurence Irving.

CHEMICAL NATURE OF THE AMPHIBIAN ORGANIZER. L. G. Barth.

SOME CONTRIBUTIONS TO THE PHYSIOLOGY OF DETERMINATION IN THE SEA URCHIN DEVELOPMENT. John Runnström.

July 17, 1934

THE CHLOROPHYL UNIT IN PHOTOSYNTHESIS. Henry I. Kohn.

A POTENTIOMETRIC STUDY OF PHTHIOCOL, THE PIGMENT OF THE HUMAN TUBERCLE BACILLUS. Eric G. Ball.

ALTERATION IN CALCIFIED TISSUES DURING DRYING (AND THEIR SIGNIFICANCE). S. E. Pond.

SOLVENT WATER IN THE ERYTHROCYTE. Arthur K. Parpart.

THE RELATIONSHIP BETWEEN THE OXYGEN CONSUMPTION AND WATER CONTENT OF THE ORGANISM. William R. Duryee.

July 24, 1934

SPINDLE FIBER ATTACHMENTS: DO THEY CHANGE WITHOUT CHROMOSOMAL INVERSIONS OF TRANSLOCATIONS? E. Eleanor Carothers.

OVULATION AND EGG TRANSPORT IN THE FROG. Roberts Rugh.

CERTAIN UNUSUAL CYTOPLASMIC ELEMENTS IN THE YOLK-SAC EPITHELIUM OF THE WHITE RAT. John W. Everett.

NOTES ON THE BEHAVIOR OF ASTERS. Henry J. Fry.

An examination of cytological literature describing normal bipolar mitotic figures (temporarily ignoring sperm-asters, cytasters, and other unusual configurations) shows that, with rare exceptions, asters occur only when two conditions are satisfied: first, when the spindle becomes organized its ends must be more or less sharply focalized; second, these focalized spindle-ends must lie in cytoplasmic areas of appreciable size. If spindle-ends arise as blunt structures, asters are absent even when the surrounding cytoplasmic areas are large; if spindle-ends are sharply focalized but lie near the cell wall or nuclear membrane, asters are again absent.

This may explain the puzzling fact that in cells of the same type, carrying on similar activities, asters may be present in those of one species but absent in those of another.

Re-investigations of a number of supposed cases of astral division show that while these asters may elongate, they do not divide; instead they disintegrate at their place of organization—the spindle-end.

Considering all types of animal cells, asters are found to be present only in a few, usually large ones. Current hypotheses concerning asters are largely shaped by the fact that most such studies concern eggs and large blastomeres, which are atypical owing to their relatively large size. Actually the majority of animal cells carry on all activities without asters.

In cases where asters are present, various facts suggest that they usually do not play a rôle in such activities as cell division or the localization of organ-forming materials. Space prevents any discussion of these points here.

It is therefore tentatively suggested, as a working hypothesis, that in normal bipolar mitotic figures, asters usually represent a secondary effect of the forces involved in forming the spindle, and probably play no rôle in cellular activities.

July 31, 1934

PERMEABILITY OF AMŒBA PROTEUS TO WATER. Coleen Fowler.

THE TENSION AT THE SURFACE OF MACKEREL OIL. J. F. Danielli.

INJURY POTENTIALS IN SCALLOP MUSCLES. H. Burr Steinbach.

THE RELATIVE EFFECTS OF INCREASED CARBON DIOXIDE AND DIMINISHED OXYGEN UPON THE HEART RATE OF YOUNG TROUT. Charlotte Haywood, Thelma O. Stevens, Helen TeWinkel, and Margaret Schott.

The effects of varied tensions of carbon dioxide and of oxygen upon the heart frequency of the fry of brown trout, Loch Leven trout, and brook trout, within 2 months after hatching, were determined. Both increased carbon dioxide tensions and diminished oxygen tensions were found to slow the ventricular beat. The time required for the heart rate to fall to one-half its original rate was taken as a measure for comparing the effectiveness of the gas mixtures used. Carbon dioxide tensions of 75 mm., 100 mm., and 150 mm. Hg slowed the ventricle to half rate in about 1 to 2 hours, 30 to 40 minutes, and 10 to 20 minutes respectively. With an oxygen tension of 80 mm. Hg, the half rate was usually not reached in 8 hours of exposure; with 25 mm. oxygen it was reached in 2 to 3 hours; and with pure nitrogen it was reached in 42 minutes of exposure.

Recovery of the heart frequency was studied following equal decrements in approximately 157 mm. carbon dioxide and in pure nitrogen. Recovery following carbon dioxide occurred gradually, but was complete between 50 and 100 minutes. Of the 17 fish subjected to pure nitrogen, 3 died; the surviving fish began recovery with a quick rebound at first, but this was usually not well sustained, and ultimate recovery did not occur until an average time of 164 minutes. These differences in recovery suggest that the mode of action of increased carbon dioxide and of diminished oxygen upon the heart frequency of the young trout is not the same.

THE HYALINE PLASMA MEMBRANE OF THE ECHINODERM EGG. Robert Chambers.

August 7, 1934

EFFECT OF CENTRIFUGING ON POLARITY IN THE ALGA, GRIFFITHSIA. Victor Schechter.

Prolonged centrifugation results in the production of a significant percentage of shoots at the centrifugal pole regardless of whether the apex or base of the cell is so oriented. If the base is centrifugally oriented the apex of the same cell may also produce shoots, but the reverse is not true. It is curious to note that the centrifuge here seems to act opposite to the force of gravity, as in nature the shoots grow upward. This fact indicates for the centrifuge a higher specificity in fractionation of materials than could occur under the influence of gravity.

PLASMODESMA IN PLANT TISSUE. L. G. Livingston.

CONCERNING THE DISAPPEARANCE OF THE EEL GRASS (ZOSTERA MARINA). Charles E. Renn.

ORIGIN AND CHEMICAL NATURE OF ORGANIC MATTER IN THE SEA WATER AND SEA BOTTOM. Selman A. Waksman and Cornelia L. Carey.

August 14, 1934

THE DIFFERENTIATION OF RAT GONAD PRIMORDIA IN NORMAL ADULT AND GONADECTOMIZED RAT HOSTS. Adrian Buyse.

THE ACCELERATION OF METAMORPHOSIS OF ASCIDIAN LARVÆ. Caswell Grave.

OBSERVATIONS ON THE FORMATION OF ARTERIO-VENOUS ANASTOMOSES. E. R. and E. L. Clark.

EXPERIMENTAL STUDY OF STRIATED MUSCLE IN VIVO. C. C. Speidel.

Striated muscle has been studied in minute detail in living frog tadpoles. In many cases the same individual fibers have been successfully kept under observation from day to day for prolonged periods of a month or more.

Growth and regeneration. In regions of rapid regeneration after tail section the entire process of histogenesis of striated muscle has been watched. This includes (*a*) the migration, proliferation, and internal movements of individual myoblasts, (*b*) anastomosis, elongation, and formation of plasmodia, (*c*) the transformation of plasmodia into young muscle fibers with characteristic cross striations (*Q*, *J*, and *Z*), (*d*) the growth in length and diameter of the young fiber and its orientation. A number of complete case histories has been obtained.

Injury and recovery. Muscle injuries have been brought about by scalding, bruising or cutting, or by treatment with alcohol, hypertonic salt solution, or other reagents. In the transition zone between normal tissue and severely injured tissue, striated muscle fibers may exhibit various grades of injury followed by recovery. Case histories are presented of the following: (*a*) recovery of a slightly injured fiber with zig-zag cross striæ; (*b*) recovery of a vacuolated fiber; (*c*) recovery of a fiber which has suffered partial loss of its cross striæ; (*d*) recovery of a fiber which has suffered total loss of its striæ without undergoing marked retraction; (*e*) recovery of a retracted fiber which has suffered total loss of its cross striæ; (*f*) recovery of a fiber after degeneration of everything except one or more muscle nuclei and some sarcoplasm.

Contraction, relaxation, and retraction. Direct observations of experimentally-induced contraction reveal the following varieties: (*a*) simple twitch without contraction band formation; (*b*) tetanic contraction with, or without, contraction band formation; (*c*) localized peristaltic-like wave of contraction, with, or without, contraction band formation (an abnormal type of contraction bearing some resemblance to that of smooth muscle); (*d*) retraction with contraction band formation (an irreversible contraction following detachment of a fiber at one, or both, of its ends).

Young, faintly striated muscle fibers, plasmodia without cross striæ, and even myoblasts are capable of contraction, though this is less rapid and powerful than the contraction of mature fibers.

These observations reveal for the first time in a living vertebrate animal the entire process of histogenesis of individual striated muscle fibers, and the steps of irritation, recovery, and repair of individual fibers following various degrees of injury. Illustrative motion pictures have been obtained.

August 21, 1934

THE TEMPERATURE-EFFECTIVE PERIODS AND "GROWTH CURVES" OF THE VESTIGIAL WINGS OF DROSOPHILA MELANOGASTER. Morris H. Harnley.

SELECTIVE FERTILIZATION IN HABROBRACON. P. W. Whiting.

MATURATION, FERTILIZATION AND CLEAVAGE OF HABROBRACON EGGS AS REVEALED BY THE FEULGEN REACTION. B. R. Speicher.

MUTATIONS AND MODIFICATIONS IN DROSOPHILA INDUCED BY SUB-LETHAL HIGH TEMPERATURE. H. H. Plough and P. I. Ives.

August 24, 1934

THE INFLUENCE OF MINUTE TRACES OF COPPER ON CERTAIN HEMOLYTIC PROCESSES. M. H. Jacobs and Samuel A. Corson.

Previous studies by the senior author have shown characteristic and striking specific differences in the time of hemolysis in isosmotic glycerol solutions of the erythrocytes of different vertebrates. In extending these studies to a large number of samples of human blood, the junior author consistently obtained times of hemolysis in 0.3 M glycerol several thousand per cent greater than those previously recorded. This striking retardation was found to be due to traces of copper in the distilled water used; it could be completely removed by redistillation from glass or by treatment of the water with Norite charcoal, and could be imitated by heating with pure distilled water a few grams of copper shavings. Copper salts in very low concentrations were likewise found to be effective. Salts of Au, Ag, Fe, Pb, Hg and Cd, on the other hand, either failed completely to inhibit glycerol hemolysis or ceased to do so at relatively high concentrations. The copper effect is a specific one, being strikingly shown with the erythrocytes of man and the rat and only slightly or not at all with those of the cat, dog, rabbit and ox. Even with human and rat erythrocytes, comparatively little effect can be obtained with other penetrating solutes such as urea and ethylene glycol. The lowest effective concentration of copper, which in some cases was found to be as low as 10^{-8} M, bears a fixed relation to the number of cells used. The reversibility of the effect may be shown by placing a very small amount of blood in a copper-containing glycerol solution and then at any desired time adding more blood. Under these conditions hemolysis not only of the added but of the originally "protected" erythrocytes promptly occurs. The same effect can also be obtained by treating a suspension of "protected" cells with Norite charcoal.

THE KERATINOID PROTEINS AND ENZYME DIGESTION. David R. Goddard.

THE THEORETICAL SIGNIFICANCE OF TALBOT'S LAW ON PHOTORECEPTION. C. P. Winsor.

CAROTINOIDS AND THE VITAMIN A CYCLE IN VISION. George Wald. Published in *Nature,* 134: 65, July 14, 1934.

August 31, 1934

THE CHROMOSOMAL COMPLEX OF THE MEXICAN GRASSHOPPER, MACHÆROCERA SUMICHRASTI. E. Eleanor Carothers.

REGENERATION IN BRACHIAL NERVES OF AMBLYSTOMA. R. L. Carpenter.

POLARISCOPIC OBSERVATIONS OF STRIATED MUSCLE OF VERTEBRATES AND ARTHROPODS. Carl Caskey Speidel.

As a result of extensive studies of striated muscle, Diamare and, more recently, Bruno have concluded that striated muscle of vertebrates differs fundamentally in optical characteristics from that of arthropods. They believe that the dark disc *Q*, which is anisotropic in arthropods, is isotropic in vertebrates, and that the clear disc *J*, which is isotropic in arthropods, is anisotropic in vertebrates. Examination of ordinary tadpole tail muscle by the writer seemed at first to confirm the claim of Diamare and Bruno. It soon became apparent, however, that a true polariscopic picture was not being obtained. The thickness of the muscle fiber, the obliquity of the Q and J discs, and their imperfect alignment caused such distortion of light with diffraction and refraction effects that the resulting image was the result of illumination by polarized light, oblique light, and (for the more superficial structures) transmitted light. Curious variations were noticeable in the exact polariscopic appearance of muscle fibers in different portions of the tadpole's tail.

Very accurate observations, however, were possible in the newly regenerating zones following partial tail amputation. In such regions, thin single isolated fibers consisting of only a few myofibrils afforded almost perfect material for polariscopic study. Distortion of light was reduced to a minimum. On such fibers examined with uncrossed and crossed nicol prisms, it could be seen clearly that the Q discs are anisotropic and the J discs are isotropic. (Delicate Z discs, showing no anisotropy, were also visible.) Illustrative motion pictures have been obtained.

The conclusions of Diamare and Bruno, therefore, are not upheld. On the contrary, clear-cut evidence from the living frog tadpole indicates that the Q and J discs of vertebrate muscle are essentially like those of arthropod muscle, Q being anisotropic and J isotropic.

A Preliminary Study of Heat Death in Marine Organisms. Paul R. Orr.

Further Analysis of the Protective Value of Biologically Conditioned Fresh Water for the Marine Turbellarian, Procerodes. Ralph Oesting and W. C. Allee.

Induced Sexual Reactions in Anura of the Woods Hole Region. Roberts Rugh.

Effect of Numbers on Rate of Cleavage and Early Development of Arbacia. W. C. Allee and Gertrude Evans.

The Nature of a Specific Egg-agglutinin Extracted from the Sperm of Arbacia punctulata. John A. Frank.

The filtrate from boiled sperm suspensions of *Arbacia* contains a substance which causes a firm, irreversible agglutination of the egg jelly of *Arbacia*. The agglutinin is contained only in living spermatozoa and is not secreted into the sea water. Sperm extracts also cause agglutination of eggs from which the jelly has been removed, dead eggs, and fertilized eggs. The agglutinin is colorless, heat resistant, is preserved on cooling to 0°, and does not give the usual protein tests. The substance is not found in any other tissues of the sea urchin. The fertilizing power of a sperm suspension ceases before the agglutinating power is lost. Time of agglutination varies inversely with concentration of the sperm extract. All attempts at artificial parthenogenesis by means of sperm extracts were negative.

Isolation of Crystalline Echinochrome. Eric G. Ball.

The tests and the eggs of *Arbacia punctulata* have been used as a source of material. Each has been worked up separately. Eggs obtained by removal of the ovaries were extracted at room temperature with acidulated 95 per cent alcohol. The pigment was precipitated from the alcoholic solution by NH_4OH, redissolved in acid alcohol, and the process repeated. The alcohol was evaporated off at room temperature and the residue extracted repeatedly with cold water to remove inorganic salts; some pigment also dissolved and was recovered by extraction with ether. The main residue was then washed with petroleum ether, in which the pigment is insoluble, to remove fats. The yield of crude pigment from 1,000 grams of eggs was 3.4 grams. Direct treatment of the shells with solvents was found to be unsatisfactory. The tests were therefore completely digested with HCl which yielded a highly colored aqueous solution and a black residue which also contained pigment. The aqueous solution was extracted with ether, the ether evaporated, and the pigment washed with petroleum ether. The black residue was treated with acidulated alcohol in the same manner as described for the eggs. The yield of crude pigment from 5,000 grams of tests was 1.1 grams.

Crystalline echinochrome was obtained by extracting an aqueous solution of crude pigment with chloroform; concentration or evaporation of the resulting solution at room temperature invariably yielded crystals. Other solvents proved less satisfactory for the production of crystals. Echinochrome obtained from tests or eggs crystallized as reddish needles or rosettes. No ash was obtained on ignition and tests for N and S were negative. Melting points could not be obtained since the material starts to sublime between 170–180° C. Crystals formed by sublimation were of the same appearance as those obtained from chloroform. The presence of a hydroxyl group in the molecule is suggested by the color reaction with ferric chloride.

RAPID DRYING OF WET MARINE TISSUES BY TWO LIQUID PHASES AND A GAS PHASE. Catherine E. Goffin and Samuel E. Pond.

NEW STEPS IN THE QUANTITATIVE DETERMINATION OF MAGNESIUM. Samuel E. Pond.

THE MYOGRAM OF THE SINGLE MUSCLE FIBER. Dugald E. S. Brown and Ferdinand J. M. Sichel.

THE CONTRACTION OF SINGLE MUSCLE FIBERS IN RELATION TO THE INTENSITY AND DURATION OF ELECTRICAL STIMULI. Ferdinand J. M. Sichel and Dugald E. S. Brown.

THE ACTION OF HYDROSTATIC PRESSURE ON TWO COMPONENTS IN A STRIATED MUSCLE RESPONSE. Dayton J. Edwards.

THE TENSION OUTPUT OF CAFFEINIZED MUSCLES. G. Saslow and E. C. Webster.

The technique of published experiments showing increases in muscular work produced by small, and decreases by larger, doses of caffeine (all below contracture level) is, for various reasons, unsatisfactory. The experiments here reported were performed upon isolated symmetrical frog gastrocnemii by a method which has been found more reliable. After an initial period of 10 isometric contractions (direct stimulation), the muscles were immersed in Ringer or caffeine-Ringer, or these solutions dripped onto the muscles, for 30 minutes; they then contracted to complete fatigue. The total tensions developed per gram muscle were computed from the record. (Those experiments were discarded in which the tensions developed in the initial period differed by more than 10 p.c.). The stimuli consisted of 0.05 second tetani at 48/minute. The muscles used (from winter *R. pipiens* and *R. palustris* and summer *R. pipiens*) weighed from 0.54–1.30 grams. The lengths varied from

3.2–4.1 cm. The caffeine concentrations ranged from 0.07 to 0.003 p.c. in Ringer. The total tensions developed in Ringer-treated muscles varied between 35 and 67 kgm. per gram muscle; in control experiments, the total tensions of symmetrical gastrocnemii in Ringer differed by only ± 3 p.c.

Over the entire range of concentrations tested, the caffeine either reduced the total tension developed or had no effect. The output of the caffeinized muscles varied between 65 (in 0.07 p.c. caffeine) and 104 p.c. (in 0.003 p.c. caffeine) of that of the symmetrical muscles in Ringer. The depressant effect was not noticeable in the first few contractions but became more and more pronounced as the stimulation continued. At and below 0.05 p.c. caffeine, the effect was reversed completely by subsequent washing in Ringer. Above this concentration, washing restored the muscles incompletely.

THE RESPIRATORY METABOLISM OF DOGFISH NERVE WITH SPECIAL REFERENCE TO POST-MORTEM CHANGE. Walter S. Root.

DETERMINATION OF BLOOD VOLUME IN THE LOWER VERTEBRATES BY THE DIRECT METHOD. Mary B. Derrickson and William R. Amberson.

The material for this study has consisted of dogfish, tautogs, bull-frogs, and eastern painted turtles.

The dogfish and tautogs were perfused with oxygenated hemoglobin Ringer solution made up with the same salt concentration as found in their blood. Using hemoglobin Ringer it is possible to keep the animals alive for some time after the experiment.

We found that oxygenated Ringer solution without hemoglobin kept the frogs and turtles alive for several hours after the blood was removed.

A normal sample of blood was drawn, after which a perfusion flow was established through a vein. The perfusion was continued until the fluid washed out clear and only a mere trace of cells appeared in the centrifuged samples.

From our experiments we found the average percentage of blood weight to body weight to be 3.71 per cent in *Squalus acanthias* (7), 1.5 per cent in *Tautoga onitis* (3), 6.42 per cent in *Rana catesbiana* (8), and 7.56 per cent in *Chrysemys picta* (6).

"LEAKAGE" AS A FACTOR IN THE ANOMALOUS OSMOTIC BEHAVIOR OF THE ERYTHROCYTE. M. H. Jacobs and H. N. Glassman.

It has long been known that the swelling of erythrocytes in hypotonic solutions, and under appropriate conditions the degree of hemolysis obtained in such solutions, is less at high than at low temperatures. An explanation recently proposed to account for this phenomenon is that at high temperatures a more rapid leakage of osmotically active materials from the erythrocyte occurs, with a consequent diminution of osmotic swelling. This explanation may readily be shown to be unsatisfactory by experiments of the following type, many of which have been made with entirely consistent results. Erythrocytes (ox, man, cat) are placed at 40° C. in an appropriate hypotonic solution of NaCl—either with or without a slight trace of phosphate buffer—which has been found in advance to produce at this temperature only a slight amount of hemolysis. After being kept at 40° C. for from 30 minutes to several hours, portions of the suspension are rapidly cooled to 20° C. and 0° C. Further hemolysis invariably occurs on lowering the temperature, the degree finally reached in each case being approximately the same as if the preliminary exposure to 40° C. had been omitted. The lowering of the temperature to 0° C. may also be carried out in two stages with essentially the same end result. If the protective action of the higher temperatures against osmotic hemolysis were due to a leakage of salts etc. from the cell it is difficult to see how after such materials had once been lost their effects could be restored by a mere change of temperature. Experiments of this type, therefore, by removing a possible alternative explanation, give support to the theory of Jacobs and Parpart that the effect of temperature on osmotic hemolysis and osmotic swelling of the erythrocyte is due chiefly to a changed distribution of base between hemoglobin and carbonic acid.

Effects of High Hydrostatic Pressure on Amœboid Movement. The Pressure Coefficient of Viscosity in Amœba Protoplasm. D. E. S. Brown and Douglas Marsland.

Amœboid Movement under Hydrostatic Pressure. Douglas Marsland and D. E. S. Brown.

Solvent Water in the Unfertilized Egg of Arbacia. A. K. Parpart and E. R. Parpart.

Effect of the Alternating Current on the Permeability of Sea Urchin Eggs to Water. Coleen Fowler.

The results herewith presented were obtained in a preliminary series of experiments made under the direction of Dr. Lucké on the effect of alternating current on the permeability of *Arbacia* eggs to water.

The set-up used was a variable lamp bank in series with the 110-volt current. At first platinum and later non-polarizable silver electrodes covered with a coat of silver chloride were used. Permeability was calculated from the changes in volume of the egg obtained from measurements of the diameter with a filar micrometer.

Some experiments were made to ascertain the effect of the current on eggs in 100 per cent sea water. In these, the current was passed through the sea water containing the eggs and the diameter of the eggs measured at one-minute intervals for ten minutes. These experiments show that the current has no effect on the volume of the eggs until it becomes strong enough to produce cytolysis. It was found that with exposure for ten seconds to 0.75 ampere current, cytolysis occurs 3 minutes thereafter accompanied by a rapid increase in volume.

The next experiments were made to find out whether these shocks have any effect on permeability to water of eggs subsequently transferred to 50 per cent sea water. In these experiments 0.7 ampere was passed through 100 per cent sea water containing the eggs in 5 closely succeeding instantaneous shocks. The eggs were then transferred to 50 per cent sea water and the volume of the eggs calculated at one-minute intervals for ten minutes. Permeability to water at the end of the third minute was calculated according to the method used by Dr. Lucké and the results obtained from measurements of 50 eggs show that permeability decreases on the average from 0.126 to 0.144. These results indicate that the effect of alternating current is to decrease permeability to water.

However, since it was thought that a more significant decrease may have been masked by the large amount of calcium present in sea water, another series of experiments was tried in which eggs were shocked in a solution of 0.9 M dextrose containing small amounts of $CaCl_2$, NaCl, and KCl, which had been shown to be isosmotic with sea water. The eggs were then immediately transferred to 50 per cent sea water and the swelling curve ascertained. The results obtained from measurements of 75 eggs show that permeability decreases from 0.121 to 0.107. It is thought that this effect will become more evident if the eggs are swollen in 0.45 M dextrose solution in the absence of large amounts of calcium and experiments to this end are being undertaken.

Further Studies on the Permeability of the Arbacia Egg to Water and to Ethylene Glycol. Dorothy R. Stewart and M. H. Jacobs.

Penetration of Acids into Sand Dollar Eggs and Yeast Cells. L. V. Beck and R. Chambers.

Changes in the Distribution of Calcium in the Arbacia Egg in Fertilization and NaCl Cytolysis. Daniel Mazia.

The Effect of Sodium and Calcium Ions on the Surface Charge of Arbacia Eggs. Katsuma Dan.

Cell Hormones and X-ray Effects on Arbacia Eggs. L. V. Heilbrunn and R. A. Young.

The Action of Ammonium Salts on the Fatty Constituents of the Arbacia Egg. L. V. Heilbrunn.

Contrasting Curves of Resistance Exhibited by Arbacia Eggs Exposed to Various Chemical Agents During the Cleavage Cycle. Anna K. Keltch, Lucille W. Wade and G. H. A. Clowes.

At last year's meeting we reported that *Arbacia* sperm was relatively highly resistant to acetic, propionic, butyric and valeric acids and sensitive to ammonia, whilst *Arbacia* eggs were relatively resistant to ammonia and sensitive to the penetrating acids. Organic bases such as ethyl amine, ethylene diamine and mono-ethanol amine show a similar effect to ammonia, being more destructive for sperm than eggs. Mineral acids, oxalic, and other strong organic acids exert an intermediary effect.

A similar differential in behavior is noted when eggs which have already been fertilized are subjected at various intervals of time after fertilization to various concentrations of each of the above-mentioned chemical agents for varying periods of time.

While previous workers have apparently concluded that all destructive agents brought to bear on the dividing egg exerted their maximum effect at approximately the same stage in relation to division, our observations indicate that this is by no means the case and that there is a definite correlation between the relative sensitivity of sperm and eggs to a given reagent and the stage at which that reagent exerts its most destructive effect on the dividing egg.

Acetic, propionic, butyric and valeric acids exert their maximum destructive effect near the peak of anaphase and just prior to commencement of telophase and a minimum effect during the resting stage or just prior to commencement of prophase. Ammonia and the organic amines exert a maximum destructive effect at or near the commencement of prophase and a minimum in telophase or early resting stage. Mineral acids like HCl, also oxalic and other non-penetrating organic acids exert a maximum destructive effect during the resting period and a minimum in anaphase or just prior to telophase. Hypotonic sea water exerts its maximum destructive effect in anaphase or the beginning of telophase and a minimum in prophase, whilst saponin exerts its maximum effect in prophase and minimum in telophase.

An additional point worthy of note is that penetrating acids exert an increasingly destructive effect and penetrating bases a decreasingly destructive effect in each successive division cycle.

ACTION OF DINITRO-CRESOL ON RESPIRATION AND CELL DIVISION IN ARBACIA EGGS. M. E. Krahl and G. H. A. Clowes.

Experiments in our laboratory and elsewhere have shown that dinitro-cresol (DNC) can raise the respiratory rate of mammalian tissues in *vitro* to 150 per cent of the normal value. We have extended these measurements, using the Warburg method, to the eggs of *Arbacia*. The results are summarized:

1. At 21° C., at 8×10^{-6} M DNC in sea water pH 8.2, the oxygen consumption per hour per unit amount of eggs (Q_{O_2}) is raised, for unfertilized, to 600 per cent of its normal; for fertilized to 400 per cent of its normal at this temperature. At concentrations above and below 8×10^{-6} M, less stimulation occurs. At 29° C., Q_{O_2} is higher for both treated and untreated eggs. The stimulation with DNC is 400 and 300 per cent for fertilized and unfertilized, respectively. This stimulation of respiratory rate by temperature and DNC is, to a very large extent, reversible. For the ratio $\frac{Q_{O_2}\text{ fertilized}}{Q_{O_2}\text{ unfertilized}}$ the values are approximately: at 21°: untreated, 4; treated, 2.9. At 29° C. they are: untreated, 2.2; treated, 1.9.

2. At 21° C. the ratio Q_{CO_2}/Q_{O_2} for both the normal and DNC-treated fertilized egg is $0.93 \pm .03$.

3. After several hours' exposure at 21° C. to 8×10^{-6} M DNC, unfertilized eggs may be returned to sea water and fertilized, subsequently developing to 90 per cent or more top swimmers. With optimum respiratory stimulation, in fertilized eggs, division can be suppressed almost entirely during the several hours of exposure to the reagent. On return to sea water these resume development at the point of interruption and develop to normal swimmers.

4. From analysis of our data on animal tissues, yeast, and marine eggs, we believe that DNC acts to promote the reduction of cytochrome or an analogous oxygen-carrier in other tissues. It does not act as an oxygen activator like methylene blue, and is dependent for effectiveness as a stimulant on the presence of activated oxygen and activated substrate. DNC does not enhance the oxidation of cysteine, even in the presence of cytolysed tissue containing traces of catalytically active metals.

EFFECT ON RESPIRATION AND CELL DIVISION OF DINITRO-CRESOL AND CYANIDE USED IN COMBINATION. G. H. A. Clowes and M. E. Krahl.

Since DNC at optimum concentration raises the respiration of fertilized sea urchin eggs to a value 400 per cent of normal and at the same time tends to suppress cell division, it appeared desirable to determine to what extent cyanide, sulfides, etc., could antagonize one or both of these effects.

A concentration of M/40,000 to M/50,000 KCN when added to the eggs before, at the same time as, or after the addition of the optimum concentration of the DNC reduced the respiration to a normal level. However, the combined effect of DNC and cyanide on cell division was not antagonistic but additive.

Since DNC and cyanide exert an antagonistic effect on respiration and an additive block to cell division, it appears probable that cell division is dependent on an oxidative process involving at least two steps. One of these may well be comparable to the dehydrase catalysed reduction of cytochrome which, as stated above, is greatly accelerated by DNC. The other may be comparable to the indo-phenol oxidase action which is known to be markedly inhibited by cyanide. The resulting disturbance of balance should not interfere with the maintenance of respiration at a normal level but might well block a sensitive mechanism like cell division which probably depends on an accurately timed series of interlocking reactions.

A cytological analysis of eggs treated with DNC or cyanide or a combination of both indicates that in the great majority of the eggs development has ceased in the prophase of the division occurring immediately after exposure to the chemical agents, or in some subsequent prophase. When returned to sea water the eggs which have been exposed for a period of 2 or 3 hours to inhibiting concentrations of either of these reagents or a mixture of the two show 95 per cent swimming forms the next day.

An oxidative process occurring in the prophase of division may lead to the formation of acid groups which in their turn may play a significant rôle in mitosis.

The above results, considered in conjunction with the data reported above regarding the action of penetrating acids and bases on different phases of cell division, lend support to the conception of alternating active acidic and resting basic phases in cell division.

Vol. LXVII, No. 3 December, 1934

THE

BIOLOGICAL BULLETIN

PUBLISHED BY THE MARINE BIOLOGICAL LABORATORY

ON DETERMINATIVE CLEAVAGE AND YOLK FORMATION IN THE HARPACTID COPEPOD TISBE FURCATA (BAIRD)

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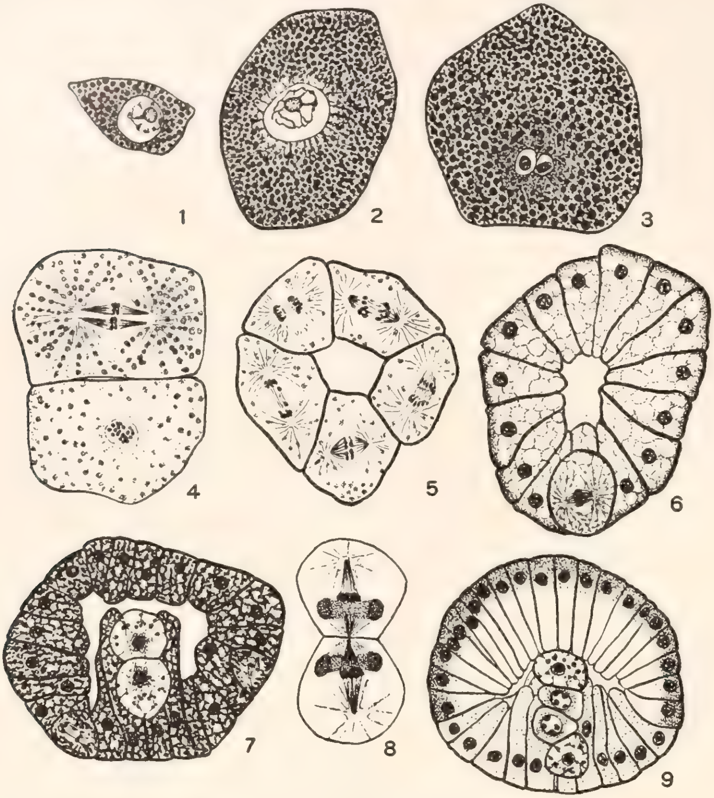
The paper by Grobben, in 1879, on the development of the crustacean *Moina rectirostris* initiated that long series of investigations on cell lineage and on formative substances which culminated in the experimental work of Boveri, F. R. Lillie, E. B. Wilson, Conklin, and many other distinguished embryologists. The crustacean egg itself, while too delicate for most experimental purposes, has been found interesting and favorable for cytological observation. Thus Kühn (1913) was able to trace the origin of the germ cells in the cladoceran *Polyphemus* from the very first cleavage division, the "Keimbahn" being marked by a cell inclusion that forms from nurse-cells during the later stages of ovogenesis. Häcker (1897) and his student Amma (1911) observed in the normal development of several species of copepods the presence of a special type of granules which collect at one end of the mitotic spindle during the early cleavage divisions. Consequently, after the fifth division the granules are transmitted to only one of the 32 blastomeres. This stem cell next divides into a primordial endoderm and a primordial germ cell, of which the latter receives most or all of the characteristic granules. These "ectosomes" of Häcker, in the present writer's opinion, may well be classed as mitochondria if the latter term is not given a too narrow and specialized definition.

Some years ago I found in one of my aquaria, among algæ collected along the shore of the harbor of Woods Hole, numbers of a large harpactid which were mostly females with egg sacs. I am indebted to Charles B. Wilson (1932) for his assistance in the identification of the species as *Tisbe furcata* (Baird). The embryological study revealed some peculiarities which seem to justify the publication of this short account.

The adult female of *Tisbe furcata* has paired ovaries which fuse with their anterior ends, dorsally above the intestine of the thoracic region (similar condition reported by Heberer (1930) for *Eucalanus*). Yolk formation in the ovocytes starts quite early (Fig. 1), and during



Camera lucida drawings. Fig. 8, × 1,000; all others × 500.

Figs. 1 and 2. Ovarial ovocytes.

Fig. 3. Newly deposited and fertilized egg from the brood sac. Note the yolk granules (black) which have attained the maximal size for the egg yolk.

Fig. 4. Second cleavage division. Yolk not represented. In this and the two following figures note the flakes of mitochondria in the dividing cells.

Fig. 5. Fifth cleavage division. Yolk not represented.

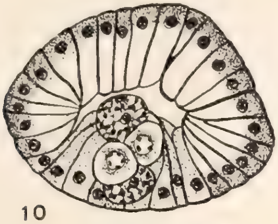
Fig. 6. Blastula of 63 cells. The stem cell near the vegetative pole just completing the sixth cleavage division. Yolk not represented.

Fig. 7. Early gastrula. Polar ingrowth of mesoderm cells carrying along the two stem cells. Yolk granules small; few in the stem cells, numerous in all other cells.

Fig. 8. Simultaneous differential division of the two stem cells. Spindles attached to the inner cell walls. Mitochondrial rings around the inner spindle cones.

Fig. 9. Gastrula. Ectodermal and mesodermal regions indicated by different stippling. Each stem cell has divided into an endodermal cell (with small yolk granules) and a primordial germ cell (with mitochondria, and vesicular nuclei). The four cells form an axial row.

the later growth of the eggs the platelets increase only in number but not in size (Fig. 2). After fertilization the characteristic double nuclei form (Fig. 3) and the first cleavage divisions are distinguished by the formation of double spindles and the maintenance of separate paternal and maternal groups of chromosomes (Fig. 4). During the mitotic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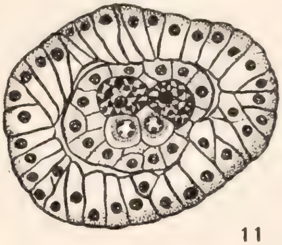
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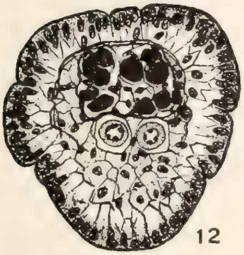
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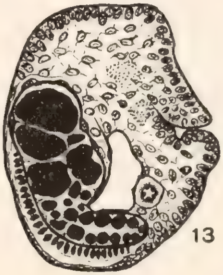
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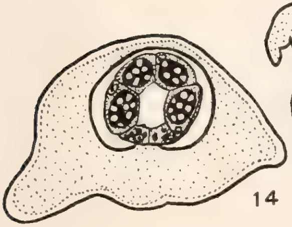
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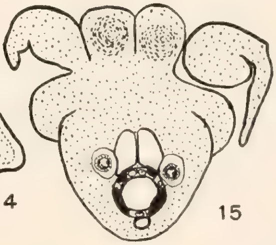
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Figs. 10, 11. Completion of gastrulation and rearrangement of the endoderm and germ cells. Yolk begins to accumulate in the endoderm cells. Ectoderm and mesoderm indicated as in Fig. 9.

Fig. 12. Frontal section through embryonic nauplius. Large yolk granules (black) in the endoderm. Two germ cells with vesicular nuclei and mitochondrial shell.

Fig. 13. Sagittal section through slightly older embryo. Extremely large yolk bodies in anterior part of the endoderm, smaller ones in the posterior section. Body cavity and ganglia are formed. Only one germ cell visible.

Fig. 14. Cross-section through nauplius near hatching stage. Yolk bodies in the walls of the gut become smaller.

Fig. 15. Frontal section through nauplius at time of hatching. Endodermal yolk nearly resorbed. The two gonocytes left and right of the body cavities.

process flakes of mitochondria appear (Figs. 4 and 5) which, however, are evenly distributed within the blastomeres. Toward the end of the sixth cleavage division one cell becomes conspicuous by its round shape and retarded division. Figure 6 shows this 63-cell stage with the round "stem cell" still in the metaphase of the sixth division. The mitochondria are evenly distributed and two equipotent daughter cells result, which have the value of left and right "stem cells." While the embryo passes through the seventh and eighth cleavage divisions gastrulation by polar immigration takes place (Figs. 7, 9, 10, 11). The "stem cells" are carried to the center of the embryo by the ingrowth of the mesoderm. Temporarily, they change from the bilateral to an axial orientation (Fig. 7). At that time the resorption of the yolk has made considerable progress. Only small granules remain in the protoplasmic meshwork. They are especially scarce in the two "stem cells" (Fig. 7). The next division is of great interest since it is qualitatively unequal, giving rise to a pair of primordial endoderm and a pair of primordial germ cells (Fig. 9). Figure 8 shows how the mitochondria, which had remained visible during the previous interkinetic phase (Fig. 7), become condensed and form rings around the inner halves of the spindles of the "stem cells." These rings are attached to the inner spindle poles by fine fibers. The subsequent unequal cell division results in the formation of two small inner cells which contain the mitochondria, and two large outer cells which contain most of the yolk granules (Fig. 9). The former are the primordial germ cells, the latter the first endoderm cells.

During the later phase of gastrulation the four cells resume a bilateral arrangement (Figs. 10, 11), the endodermal cells occupying a position antero-dorsal to the germ cells. The germ cells remain without further divisions up to the time when the nauplius larva hatches from the egg sac (Fig. 15). They are easily identified by their large size, large nuclear vesicle with compact chromosomes, and by the dense shell of mitochondria around the nucleus.

Of great interest is the behavior of the endoderm cells, which multiply by mitotic division and give rise to the midgut, as indicated in Figs. 12 to 15. During the early phase of nauplius differentiation they exhibit a peculiar process of yolk formation (Figs. 11–13). The rapidly growing yolk platelets soon begin to coalesce, resulting in the formation of about eight large and many small grains. Some of the endodermal cells become greatly inflated, assuming the function of a dorsal yolk sac in the embryonic nauplius (Fig. 13). Recent students of yolk differentiation have concentrated their attention on the synthesis which takes place during ovogenesis. Although given little attention, endodermal yolk formation as observed in *Tisbe* is widespread among crustaceans.

It is closely related to the "vitellophage" process by which the endoderm of the crayfish *Potamobius* absorbs the uncleaved yolk mass of the egg (Reichenbach, 1886). The only difference is that in the former species the *egg yolk* during cleavage is evenly distributed in all blastomeres. As in the crayfish this primary yolk becomes liquified and when redeposited as *endodermal yolk* it forms grains of strikingly different size. It may be added that the endodermal yolk gives the same reactions with eosin, acid fuchsin, iron hematoxylin, and other dyes as the yolk of the ovarial egg and the early blastomeres.

If compared with cladocerans the development of the copepods shows many striking similarities. However, the "stem cell" appears at the close of the third cleavage division (Grobben, 1879; Kühn, 1913) and divides during the fourth into one endoderm and one germ cell. According to McClendon's (1907) description of the development of parasitic copepods, it seems that the "stem cell" is formed at the close of the fourth cleavage. In non-parasitic copepods, however, the "stem cell" is formed only by the fifth cleavage. In *Cyclops fuscus, Diaptomus cœruleus,* and other species investigated by Amma (1911) the sixth division has been found to be segregational, while in *Tisbe furcata* it produces a pair of equal left and right "stem cells." The seventh division consequently is equal in *Cyclops* and *Diaptomus,* but segregational in *Tisbe,* thus producing in all genera one pair each of endoderm and germ cells. The postponement of the segregational division in *Tisbe* coincides with the practically equal distribution of mitochondria in the early cleavage stages of this species. We do not intend to reopen the discussion about the significance of the mitochondria or "ectosomes." However, it is evident that mitochondria by their peculiar distribution, if not responsible directly for the segregation of endoderm and germ plasm, indicate at least a polar differentiation within the cytoplasm of the stem cells, even before the beginning of the differentiating division.

Summary

In the development of *Tisbe furcata,* a harpactid copepod of the Woods Hole region, there occur two periods of yolk formation. Egg yolk is accumulated in the usual way during ovogenesis. After gastrulation and during the differentiation of the nauplius, yolk is deposited again in the endoderm, which partly assumes the character of a yolk sac.

As in other non-parasitic copepods, endoderm and germ cells originate from a common "stem cell" of the 32-blastomere stage. *Tisbe furcata* is peculiar in that an equational division precedes the one that separates the endodermal from the germinal line. In the stem cells the

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segregational division is prepared by a polar differentiation of the cytoplasm which is most clearly indicated by the asymmetrical distribution of the mitochondria.

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FURTHER OBSERVATIONS ON THE POLARITY OF THE EGGS OF URECHIS CAUPO

VESTA HOLT

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The eggs of *Urechis caupo*, an echiuroid worm recently described by Fisher and MacGinitie (1928), are found in abundance at several readily accessible points along the coast of California. Owing to their abundance, size, and transparency, and to their unusual viability under ordinary laboratory conditions, they are proving to be valuable material, especially for the experimental embryologist. It is therefore essential that their normal embryology be known, and of first importance that a definite mark of polarity in the immature eggs, if present, be recognized.

The results of a series of checks upon the polarity of normal and centrifuged eggs of *Urechis,* with the conclusion that the immature egg bears no visible mark of polarity, were published by Taylor in 1931. Earlier in the same year, Tyler (1931) presented evidence that the indentation, which is characteristic of these eggs, marks the animal pole. Later, this author (Tyler, 1932) published further data and concluded that " The main question involved in this work is whether or not the polarity of the *Urechis* egg is determined before fertilization, as appears to be the case for most animal eggs. For the type first discussed the results clearly show that polarity is already established in the unfertilized egg, and the pole is marked by the position of the indentation. For the type in which a second indentation appears after fertilization there is no reason to doubt that polarity is established before fertilization although the position of the pole is not so clearly marked by the first indentation as by the one appearing after fertilization."

According to this author, then, there are two types of eggs in *Urechis* as regards the point of extrusion of polar bodies: (1) those wherein this extrusion coincides with an indentation *before* fertilization, and (2) those in which, *after* fertilization, a second indentation forms and corresponds with the point of extrusion of polar bodies. Just what relation this second indentation has to the one in immature eggs is not made clear so that immature eggs of this second type apparently have no mark of polarity.

Because of the discrepancies between our findings of 1931 and those of Tyler (1931, 1932), we undertook to recheck our former results and to make further studies on the polarity of this egg for comparison with Tyler's data.

For this purpose, eggs were used from fourteen females. Three of these, viz., 1, 2, and 3 (see Table I), which were used during July and August, 1931, were dug at Morro Bay on July 18, then taken directly to Pacific Grove in a glass jar of sea water which was kept cool in a bath of ice water, and placed in aquaria of running sea water. The studies on the eggs of these females were made from July 20 to August 10.

The eleven females, *A* to *K* inclusive, and the male *A* were dug within an area of one square yard at Elkhorn Slough on December 18, 1931. These were taken to the laboratory in jars containing water from the slough and transferred within two hours to aquaria with running sea water. The *ME* female was one of the three from Morro Bay which had been used the previous summer and left in the aquarium of running sea water until December, when further studies were made on the eggs, as indicated in Table I.

The vaseline slide method recommended by Tyler (1931) was used. For recording the location of polar bodies, the surface of the egg was roughly divided into three regions. Region I is the 90° containing the indentation and corresponding approximately to its size; Region II is the equatorial band; and Region III comprises the 90° of area opposite the indentation. Also a check was made of the eggs which developed polar bodies within 10° of the center of the indentation.

Many eggs show two or more indentations. It may be that many of those considered above have another indentation up or down, but none was recorded which showed more than one. In the following tabulation, polar body formation in eggs showing two indentations is recorded. These eggs were taken from various females and the check made along with the previous checks.

In the smaller indentation	25 eggs	38%
In the larger indentation	10 "	15%
In neither indentation	31 "	47%
Total	66 eggs	100%

Microphotographs which were made of many of these eggs definitely show the same points listed in these tables.

In June, 1934, a more carefully controlled study was made on a small number of eggs. Each egg was rolled over by means of a fine jet of water from a micropipette and the entire surface examined. Only those eggs were chosen which showed one indentation. Each egg was kept under *constant* observation for the first 15 minutes after insemination and in several cases until polar body formation. The slides were never removed from the microscope until after maturation.

Two groups of females were used. The first group had been dug at Elkhorn Slough May 10 and had been used during the interim to supply eggs for other experiments. The females of the second group were also from the slough but were freshly dug at the time of the study, June 17. The results are given in Table II.

TABLE I

Females	Dates	Temp. range °C.	No. eggs with polar bodies in Region I	No. eggs with polar bodies in Region II	No. eggs with polar bodies in Region III	No. eggs 0°–10° divergence	No. eggs 10°–180° divergence
1	7/20–8/6	18½–20½	58	19	9	44	40
2	8/5–8/10	19 –20½	25	46	11	17	65
3	8/6	19½–20½	2	5	3	1	9
A	12/21, 29	15 –18	20	37	10	11	52
B	12/22–1/1	14½–18½	17	27	14	9	49
C	12/23	14 –16	6	6	5	2	17
D	12/24	15½–17½	6	6	3	3	13
E	12/24	17 –17½	4	11	3	4	14
F	12/25	16 – 16	3	13	2	1	17
ME	12/26, 27	16 –18½	12	11	7	11	20
G	12/26, 31	13 –17½	8	7	2	9	15
H	12/28	18 –19½	3	15	3	2	20
I	12/29	15½–16½	13	13	3	10	19
J	12/30	18 –18½	4	12	1	5	13
K	12/31	17 –18	7	4	1	6	6
Totals			188	232	77	135	369
Percentage of total number of eggs			38	47	15	27	73

Careful observation of the egg following insemination reveals a fairly regular cycle of events. Original indentations disappear between

2 and 4 minutes, the membrane appears in approximately 5 minutes, and secondary indentations appear from 6 to 9 minutes. There may be *one or many* of these secondary indentations. They may appear within the area of the original or at any point on the surface of the egg. They usually remain from 4 to 9 minutes. Fifteen out of forty eggs formed polar bodies at the location of a secondary indentation, but thirty-eight such indentations (in twenty eggs) were observed which bore no relation to the point of extrusion of polar bodies.

TABLE II

	Old Worms	Fresh Worms
Polar body forms in original indentation	10	2
Polar body forms in a secondary indentation within area of original	2	3
Polar body not in area of original indentation but		
1. Where there has been no indentation at any time	1	1
2. Where a secondary indentation was observed to form after insemination	5	8
3. On the upper or lower surface of the egg where there was no original indentation but where a secondary may or may not have formed	3	7

From these observations it appears that the behavior of the indentations in *Urechis* eggs may be a purely physical phenomenon, related to permeability and viscosity changes following fertilization. Centrifuging results show a marked fall in viscosity at fertilization. This is no doubt due to the absorption of water which causes the indentations to fill out. Then there is a reversed process. Measurements of eggs show that at fertilization there is only a slight membrane elevation but that the perivitelline space results primarily from a shrinkage of the egg away from the membrane. Accompanying this shrinkage are numerous bucklings and the formation of temporary, secondary indentations over the surface of the egg.

Whatever the explanation for these various changes on the egg surface may be, two points are evident from the results published by Taylor in 1931, those of Tyler in 1932, and those given above:

1. That polar bodies do not *always* appear at an indentation found in the immature egg. Hence this cannot be used as a mark of polarity.
2. That polar bodies do not appear at all secondary indentations of the fertilized egg. Hence a specific, secondary indentation cannot be used as a reliable mark of polarity, even *after* fertilization.

Therefore we maintain our original thesis, viz., that there is no reliable mark of polarity on the immature egg of *Urechis caupo* and until such a mark can be located with certainty, it cannot be said that the polarity of this egg is, or is not, established prior to fertilization.

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ACCESSORY HEARTS IN THE OYSTER, OSTREA GIGAS[1]

A. E. HOPKINS

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It is well known that in some invertebrates certain peripheral blood vessels are specialized to pulsate and pump blood to organs which otherwise would receive very little circulation. The pulsating ampullae at the bases of the antennae of some insects are frequently referred to as examples of peripheral accessory hearts. (See von Buddenbrock, 1928.) More widely recognized are the "gill hearts" of cephalopods, though it appears that similar structures have not been identified in other mollusks.

During a study of the mechanism by which the Japanese oyster (*Ostrea gigas*) pumps water, a number of specimens were prepared for observation by sawing away portions of the right, or upper, valve. Generally the free edge of the mantle folded back over the cut surfaces, where it started to secrete new shell. In such cases the mantle chamber, in which the gills hang, and the cloacal chamber, from which exhalant water is discharged, were exposed. In these oysters a pair of well-defined, actively pulsating vessels was observed. It appears that, due to failure to observe these organs, some authors have not understood the circulatory system of the oyster entirely correctly.

In the expanded condition the accessory hearts appear as prominent vessels along the inner surfaces of the mantle folds which form the lateral walls of the cloacal chamber (Fig. 1). Distally they end near the margin of the mantle, slightly forward of the septum which connects the two mantle lobes and to which the posterior ends of the gills are attached. Centrally they disappear into the excretory organs (organ of Bojanus) adjacent to the ventro-lateral borders of the adductor muscle. In this manner they cross the cloacal chamber in a diagonal direction.

Dissections after injection showed that apparently a thin septum separates the blood spaces of the excretory organ from veins entering the auricles. If, as appears from these observations, the spaces of the kidney do not open directly into the auricles but are forced to dispose of the blood collecting in them from the viscera in some other manner, it is necessary to modify our concept of the mode of circulation. The gills are apparently supplied only with venous blood from the organ of Bojanus while such blood enters the mantle along with arterial blood.

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It was stated by Leenhardt concerning the Portuguese oyster, *Gryphea (Ostrea) angulata* (1926, p. 82) that: "Les branchies ne reçoivent que du sang provenant de l'organe bojanien, mais les lacunes de cet organe communiquent avec une grosse veine afférente du coeur qui peut ramener directement le sang à l'oreillette sans passer par la branchie." He considered that the circulatory system of the gills is "adrift" on the lacunae of the excretory organ which has a much larger vessel leading into the heart, so that there would be little pressure to force blood through the gills. However, he did not observe the accessory hearts.

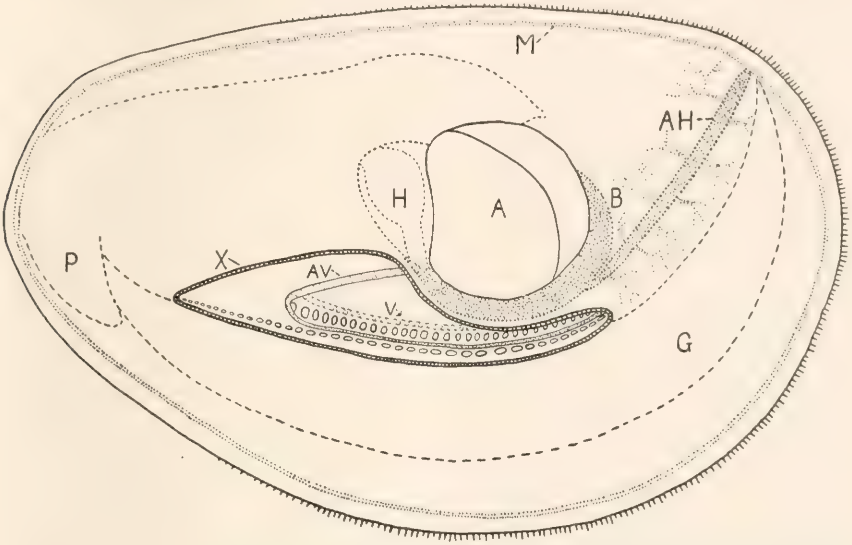

FIG. 1. Diagrammatic view of oyster from left side with part of mantle removed along double line (*X*). *A*, adductor muscle; *AH*, accessory heart (which is actually on inner face of mantle); *AV*, afferent vein; *B*, organ of Bojanus; *G*, outline of underlying gill; *H*, heart; *M*, marginal artery of mantle; *P*, labial palp; *V*, medial vein forming insertion of the two medial demibranchs.

Although not previously recognized as active organs, Kellogg (1890, Fig. 9) shows them in a figure of a cross-section through the cloaca, but refers to them only as ridges "running backward on inner wall of mantle, continuous with line of concrescence of gill to mantle." He failed to identify them as vessels since they were probably fixed in a state of contraction.

It proved difficult to inject the accessory hearts of a living specimen because of their marked sensitivity to mechanical stimulation. Even in an oyster anaesthetized with magnesium sulphate the slightest stimulation of the vessels caused them to contract. By leaving oysters in a dilute solution of ammonium bromide in sea water for 24 hours, after which they were wide open and the adductor muscle completely relaxed, and then adding alcohol up to between 5 and 10 per cent, specimens were obtained in which all blood vessels, including both the heart and the accessory hearts, were completely expanded and insensitive to irritation. These were removed from the shell by cutting the adductor muscle close to its attachment without damaging the mantle. Injections of three kinds were made with suspensions of carmine or ink in sea water: into the ventricle; into the auricles, with the ventricle tied off; or into either of the accessory hearts. A fine cannula was used to inject the accessories so that the fluid could flow in both directions.

The Circulatory System

Complete description of the anatomy of the circulatory system is not necessary here, for various investigators (Leenhardt, 1926; Kellogg, 1890) have published such studies on *O. virginica* and *O. angulata*. However, it is necessary to describe the circulatory system in general in order to arrive at an understanding of the function of the accessory hearts.

When the ventricle is injected, the fluid enters the adductor muscle through the posterior aorta, and the viscera, labial palps, and mantle through the anterior aorta. The anterior aorta runs forward near the dorsal surface of the visceral mass, giving off along its course several large arteries to the visceral organs. At the anterior end it opens into a large vessel running dorso-ventrally adjacent to the hinge ligament of the valves. From this vessel there are five main branches, the first of which goes to the palps. At both dorsal and ventral extremities it divides into a pair of pallial arteries which run around the margin of the mantle. The ventral pallial artery of each lobe is continuous with the dorsal pallial artery of the same side through the marginal artery, which makes a complete circuit of the mantle lobe. The marginal artery sends small branches distally to the border of the mantle and tentacles, and large ones proximally into the tissues of the mantle proper.

Blood is collected in the lacunae of the mantle and carried directly into the auricles. When either the ventricle or the auricles are injected, the fluid enters neither the kidneys nor the accessory hearts. Because of the fact that the venous system consists of blood spaces which generally lack definite walls it is most difficult to trace the course of blood returning from the tissues. Apparently blood from the viscera, palps, gills and adductor muscle enters a large vein which forms the line of union of the two medial demibranchs. This vein is enclosed within the ventral wall of the visceral mass from the anterior end of the gills to a point below the adductor muscle, posterior to which the gills hang free. Veins from the lateral demibranchs enter the medial vein by way of the septa which separate the gill lamellae into canals. On either side, about halfway between the adductor muscle and the anterior end, a vein runs from the corresponding gill into the lacunae of the excretory organ.

By injecting into one of the accessory hearts so that the fluid could proceed in both directions it was found that it entered not only the kidney but also the gills and the marginal arteries of both lobes of the mantle, as well as the other accessory. This was most surprising since the marginal arteries had been shown to receive direct arterial blood from the anterior aorta. It appeared to mean that the accessory hearts, by pumping blood from the kidneys into the marginal arteries, are forced to work against the arterial pressure already in the arteries.

That this is the case was shown by injecting carmine suspension into the ventricle until both marginal arteries, and branches from them into the mantle, were well penetrated. Then a suspension of blue ink was injected into one of the accessory hearts. Traveling distally the blue fluid entered the marginal arteries of both mantle lobes, crossing from one to the other through the connecting septum, and forced the red fluid back anteriorly. The blue replaced the red suspension in the arteries, and the branches from them into the mantle showed red in the finer subdivisions with blue in the trunks. Along its course each accessory heart gives off numerous small branches directly into the adjacent portions of the mantle.

Centrally the accessory hearts are continuous with the excretory organs, from which they receive blood. The organ of Bojanus is a rather indefinite structure within the mantle, extending laterally from the posterior surface of the adductor muscle around the ventral side of the muscle to a point below the pericardium. It consists of finely divided blood spaces in which the excretory tubules are suspended. A colored fluid forced anteriorly from the accessory heart passes with difficulty through this organ and into the veins or lacunae which collect blood from the viscera and adductor muscle. Some confusion was caused by the fact that in certain cases the fluid also penetrated the auricles when this injection was made, but it was found that this occurred only when the fluid was injected with considerable force.

It would appear that the accessory hearts play a very important rôle in completing the blood circulation through the excretory and respiratory organs. The mantle of lamellibranchs, with its rich supply of superficial blood spaces, is considered to be the primary organ of respiration, while the gills function chiefly to pump water from which they extract food particles and transmit them to the mouth (Galtsoff, 1928; Nelson, 1923; Hopkins, 1933). The gills receive all of their blood, and the mantle at least a part of its supply from the excretory organ, and the blood from the latter is returned directly to the auricles.

Activity of the Accessory Hearts

In a large specimen the activity of the accessory hearts is most conveniently observed. Japanese oysters from 15 to 20 cm. long were found to have large, well-developed accessory hearts which could be readily studied. In such a specimen they may be observed as a pair of long vessels on the inner surface of the mantle, within the cloacal chamber, which when contracted are not more than 1 mm. in diameter, but which expand, as they fill with blood, to a diameter as great as 5 mm. They may be visible for a length of 2 to 4 cm. so that waves of contraction are easily seen. When a specimen is opened, by cutting the adductor muscle, the accessory hearts, like the heart itself, do not function normally but waves of contraction run in all directions. If the specimen be placed in running sea water for a few hours they begin to pulsate in an orderly manner.

In order to observe with certainty the normal activity of these organs, however, a method was employed whereby it was not necessary to cut the adductor muscle or otherwise injure the specimen. Portions of the right, or upper, valve were carefully sawed away in such a manner as to avoid injuring any underlying tissue. The free parts of the right mantle lobe characteristically would fold back over the cut edge of the shell, exposing the mantle chamber and cloaca. Through the latter chamber the pericardium could be seen and the rate of pulsation of the heart observed while at the same time the accessory hearts were exposed to view. Studies on such an oyster should result in an understanding of the normal activity of the organs, for the circulatory system was completely intact.

It was not feasible to record the pulsations of the accessory hearts by the kymograph method, for they are extremely sensitive. A pulsation begins, when the organ is fully expanded, as a constriction at the central end, next to the kidney, and extends slowly outward. The direction of the wave is very distinct. If the distal end be stimulated mechanically at the time a wave of contraction is starting at the proximal end the result, for several minutes, is a confusion of ineffective waves of contraction passing in both directions. An unsuccessful attempt was made to observe blood cells within the accessory hearts so as to be sure that the blood flow is from the kidney to the mantle.

In order to determine whether a definite relationship exists between the rate of heart-beat and the rates of pulsation of the accessory hearts, counts were made consecutively of the time required for a given number of pulsations by each. In Table I a typical series of such measurements is given, showing that the three organs need not beat at the same rate. The accessories pulsate at a rate only one-third to one-fourth that of the heart, and the two accessories do not act either in unison or at the same rate.

TABLE I

Comparative rates of pulsation of the heart and the two accessory hearts

Time	Temperature	No. Pulsations per Minute		
		Heart	Right accessory	Left accessory
	°C.			
9:25	17.2	15.7	2.5	6.3
9:29	17.3	16.1	4.5	6.2
9:33	17.4	16.8	5.0	7.1
9:57	18.1	17.9	4.5	8.0
10:01	18.2	17.1	5.1	7.1
10:05	18.3	17.7	4.8	8.0
10:46	19.4	20.1	5.3	8.0
11:20	19.8	21.3	6.4	8.6
2:34	19.9	18.5	7.6	6.6
2:38	19.9	16.5	6.5	6.5
3:03	18.8	17.9	6.6	7.6
3:06	18.8	18.6	6.9	7.2
3:56	18.5	16.8	6.1	9.0
3:59	18.5	17.3	5.5	6.4
4:20	18.5	18.2	9.9	9.1
4:23	18.5	18.2	8.9	9.1
Average		17.8	6.0	7.5

The results suggest that the rate of pulsation of the accessories is determined only by the rate at which they fill with blood, and that in this respect only is their activity dependent upon that of the heart. In further support of this assumption is the fact that typically the left accessory pulsates more rapidly than the right, which may be due to the oyster's position upon its left, or cupped valve, so that gravity causes the lower, or left, accessory to fill with blood more rapidly.

COURSE OF CIRCULATION

The circulatory system of the oyster, as has been stated above, must be looked upon as consisting of two incompletely separated parts (Figs. 1 and 2). The heart pumps blood through the arteries to the adductor muscle, labial palps, and viscera, from the lacunae of which it is collected and carried to the excretory organs. The heart also pumps blood directly into the marginal arteries of the mantle lobes. From the excretory organs the accessory hearts force the blood into the gills and into the marginal arteries, which therefore receive both arterial and venous blood. It is remarkable that such large vessels as the marginal arteries should receive both types of blood and, since the lacunae of the mantle carry blood directly back to the auricles, the heart receives a mixture of blood only a part of which has passed through the excretory organs. A marginal artery is a complete loop, into one side of which the heart pumps arterial blood, while the accessory hearts pump venous blood into it at the opposite side. The loop, then, is subjected, along its course through the mantle, to arterial pressure from both directions as well as to venous pressure from the accessory hearts. This pressure presumably forces the mixed arterial and venous, purified and unpurified, blood through the smaller vessels of the mantle, assuring aëration by means of the current of water produced by the gills.

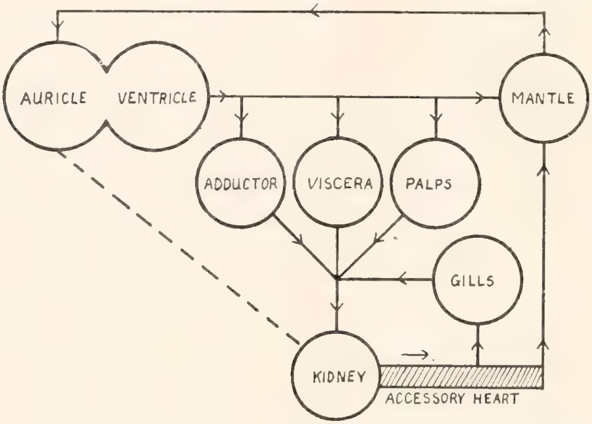

Fig. 2. Diagram of the course of circulation in the oyster showing part played by accessory hearts.

A schematic diagram of the apparent course of circulation is presented in Fig. 2. Because of the difficulty of tracing the veins in the vicinity of the excretory organs it is uncertain whether or not the blood spaces of these organs are actually continuous with the auricles. On the other hand, the veins from the mantle may be traced directly into the auricles.

This interpretation differs in some respects from the description of *Gryphea (Ostrea) angulata* given by Leenhardt (1926, p. 82): "Le sang provenant de la masse viscérale passe tout entier par l'organe excréteur. Il n'en est pas de même du sang revenant des palpes labiaux qui se déverse directement dans les vaisseaux ramenant le sang à l'oreillette; de même le sang revenant du manteau est en grande partie déversé directement dans les veines afférentes du cœur." Kellogg (1890, p. 408) described the lamellibranch venous circulation as follows: "From the irregular sinuses into which the arteries empty, the blood is collected in larger vessels and conveyed to a vessel beneath the pericardium, called the sinus venosus. Thence it passes to the gills, traversing on the way the walls of the nephridia, where waste products are excreted. The circulation is completed by the return of the blood from the gills to the auricles of the heart."

Apparently veins from the visceral mass, palps, and muscle open into the large medial sinus (Fig. 1, *V*) which forms the insertion of the two medial demibranchs. By injecting a colored fluid into this sinus the small vessels of the gill filaments are penetrated, showing that the efferent blood from the gills is not kept apart from the rest of the venous blood. This vein communicates with the longitudinal vein of each gill through the connectives separating the gill into canals. From each longitudinal vein (Fig. 1, *A.V.*) a large vein runs to the venous sinus which communicates directly with the excretory organ. The sinus may also open into the auricles, though it is difficult to be sure. On the other hand, the veins returning blood from the mantle to the auricles are well defined and may readily be traced.

The marginal vessels of the oyster appear to be entirely different from those of the mussel, as described by Field (1922) and others, and the course of the circulation in the two cases is decidedly different. According to Field, the aorta of the mussel runs anteriorly along the dorsal surface of the viscera, giving off branches to the mantle lobes. Around the border of the mantle runs the marginal sinus, which collects blood from the mantle and carries it through a large vein to the kidney, from which it passes directly into the heart. The mantle then receives only arterial blood. With the blood spaces of the kidneys communicating directly with the auricles by a large vessel, it may be considered that accessory hearts are not necessary, that without such assistance the circulation of the blood is completed.

It may be that with respect to the accessory hearts oysters represent an exception among lamellibranchs, though it would be of interest to determine definitely whether this is the case. Grave's (1909) description of the wing-shell, *Atrina,* suggests that accessory hearts may be found in this species also. He stated (p. 427), "After bathing the glandular cells of the kidney the blood is collected into a large vein which transports it to the gills to be aërated." He apparently did not observe pulsations of this vein, though such an observation would be of interest.

Among mollusks it would seem that the only structures known which have a function comparable to the accessory hearts of the oyster are the gill hearts of cephalopods. These organs have long been known as remarkable examples of pulsating blood vessels. Their function is to pump blood from the excretory organ, where it is collected after passing through the viscera, to the gills, from which it returns to the heart. The accessory hearts of the oyster appear to be homologous to the cephaloped gill hearts in that they take origin in the vessels of the kidneys and pump blood to the respiratory organs, in this case primarily the mantle and secondarily the gills. In both groups the function is the same; namely, to return venous blood from the excretory organ to the auricles by way of the organs of respiration. The term, *gill hearts,* might also be used to refer to these structures in oysters.

Although all of the observations described above were made on *Ostrea gigas,* it was determined that both *O. virginica* and *O. lurida* possess similar pulsating accessory hearts. The structures appear to be characteristic of the genus.

Summary

In three species of *Ostrea* a pair of actively pulsating accessory hearts was observed in the lateral walls of the cloacal chamber. They appear to pump blood from the lacunae of the excretory organs into the large marginal arteries of the mantle lobes and into the gills.

Counts of the rate of pulsation of heart and accessory hearts in *O. gigas* show that they act independently, the rate of pulsation of the accessories probably being determined by the rate at which they fill with blood. Only in this respect is their rate dependent upon the heart rate.

The accessory hearts appear to be homologous to the gill hearts of cephalopods.

The course of circulation, as influenced by the accessory hearts, is apparently as follows: blood from the ventricle passes through large arteries to the viscera, adductor muscle, and palps; the anterior aorta is directly continuous with the marginal arteries of the mantle; venous blood from viscera, palps, gills, and muscle passes to the excretory organs from which it is pumped by the accessory hearts into the marginal arteries of the mantle and to the gills; blood collected from the mantle returns directly to the auricles.

Heart and accessories both pump blood into the marginal arteries, which therefore receive both venous and arterial, purified and unpurified blood.

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MODIFICATION OF BEHAVIOR IN NEREIS VIRENS

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In a recent paper (Copeland, 1930) the senior writer described an experiment on *Nereis virens* which indicated that the worm formed a conditioned response to both increased and decreased illumination. The results were made possible through the fact that *Nereis* will remain in a glass tube placed in sea water, and there behave essentially as it does in its natural burrow in the sand.

Under laboratory conditions one of its most interesting reactions is that to food (Copeland and Wieman, 1924). If, for example, a fragment of clam is dropped or held at the end of a tube occupied by a worm carrying on its characteristic undulatory movements of the body (Fig. 1, *A*), juices from the clam are drawn into the tube which stimulate the animal. Its response is a forward movement through the tube continued, often rapidly, until it arrives at the end (Fig. 1, *B* and *C*), when the food is seized. If the observer desires he may feed the worm by holding the piece of clam in forceps as illustrated in Fig. 1, *H, I,* and *J*. When, however, the food is removed after stimulation occurs the worm, on coming to the end of the tube, in most instances extends the anterior portion of the body outward, swinging it to the right or left or to the surface of the water in an attempt to find the clam. If the animal is particularly positive to the food stimulus it may stretch far outward and carry on active swaying and turning movements for a period of several minutes before withdrawing into the tube. These searching movements, as we shall designate them, are extremely characteristic of *Nereis* under food stimulation, and are readily recognized in various degrees of intensity by one who is at all familiar with the behavior of the species. Some of the positions assumed by a worm at this time are shown in Fig. 1, *D, E, F,* and *G*. It is this remarkably active and definite reaction of *Nereis* to food substances that gives the experimenter a favorable opportunity to test its ability to form a conditioned response. Having studied the worm's behavior when a suddenly modified illumination was used to signify the presence of food, it appeared of interest to try in a similar way a tactile stimulus to which the animal usually is more negative than positive.

Three worms, collected October 14, which will be designated *A, B,*

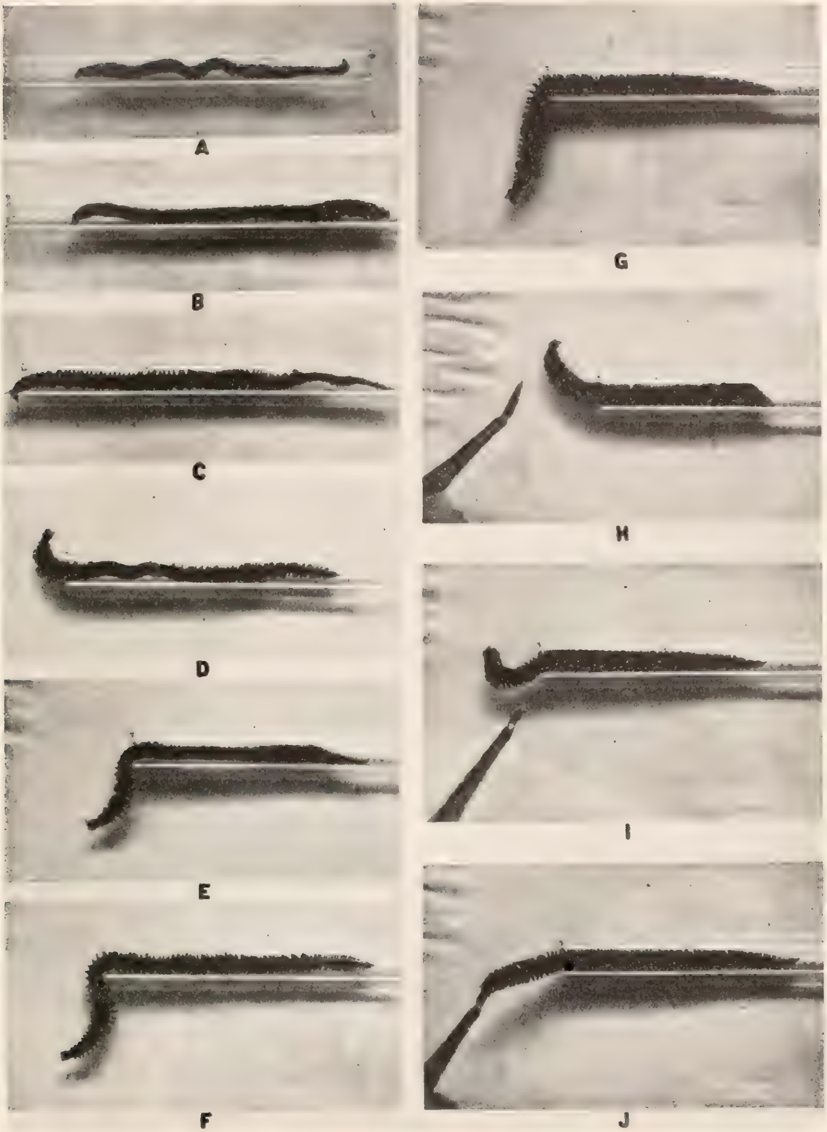

FIG. 1.

A. Nereis virens in a glass tube carrying on undulatory movements of the body which draw the sea water through the tube.

B. Worm moving forward after being stimulated by materials emanating from a fragment of clam held for a few moments at the end of the tube.

C. Worm starting to advance beyond the mouth of the tube.

D, E, F, G. The anterior end of the body, extended several centimeters out of the tube, is moved in various directions as the worm attempts to find the food which has been taken away. The animal is now exhibiting typical searching movements.

H, I. A piece of clam, held in forceps, is being offered to the worm.

J. Worm taking the clam from the forceps.

and *C*, were selected for the experiment. On being brought into the laboratory they occupied glass tubes which were placed in shallow square dishes of Pyrex glass, measuring about 21 cm. across the bottom and 3 cm. in height, containing sea water to the depth of about 2.25 cm. The tubes, approximately 10 cm. in length, with inside diameters of slightly over 5 mm., were large enough to allow the worms to carry on undulatory movements of the body and to turn around without difficulty.

The dishes, arranged in a row 8 cm. apart, were placed on a support painted white so that they paralleled the base of an east window 88 cm. distant. The buff-colored shades of this window, and another on the same side of the room, usually were pulled down to within 12 cm. of the bottom to protect the worms from the direct rays of the morning sun. Since there were no other windows in the room, the experimenter could carry on his work without shading the animals.

A small stone resting on each tube held it in position 6 cm. from the side of the dish nearer the window and parallel to it.

Beneath the dishes and tubes centimeter scales were marked off, and at both ends of the tubes three concentric circles were traced with radii of 1, 2, and 3 cm. By this method of graduation it was possible to record at any time the position of the worm in the tube, or its progress outward.

In order to keep the physiological state of the animals as near normal as possible, the sea water was changed about every four days and the dishes carefully cleaned in tap water. At no time was a worm tested within four hours after the water had been renewed.

The room temperature during the experiment varied between 14° C. and 28° C., but no resultant modification in the behavior of the animals was noted.

On October 24 ten preliminary tests were begun to determine the reactions of the worms to a tactile stimulus. To effect this stimulation a delicate, blunt-ended glass rod was used. It was bent at a right angle about 2.5 cm. from the end in order that it might easily be inserted into the tube containing the worm. Great care was taken to keep the rod perfectly clean. The end with which the worm was touched was not handled, and before and after each test it was washed in distilled water and dried with filter paper. If the worm was completely inside the tube the rod was carefully inserted and the animal's tentacular cirri, or other portions of the head region, were lightly touched. Immediately afterward the rod was withdrawn and the behavior of the worm recorded over a period of two minutes.

In order to avoid the possibility of the worms forming an association between touch and food in the preliminary trials, they were fed and given tactile stimulation on alternate days. At every feeding they received four pieces of clam 2 or 3 mm. in diameter. These were always placed at that opening of the tube toward which the animal's posterior end was directed. After the worm turned about it usually began wave-like movements of the body drawing sea water and juices from the clam into the tube. By this method, therefore, the worm was stimulated chemically in most, if not in all, cases before there was any contact with the clam whatsoever.

On the days when the worms were touched with the glass rod they were tested usually twice, once in the morning and again in the afternoon. Occasionally, however, there was but one trial during the day.

The reactions to the rod were all graded from 10, the most positive, to 0, the most negative. These eleven types of response may be briefly described as follows:

10. Worm moves forward at once, extending its anterior end out of the tube. The head is moved about in various directions, just as it is when the animal is chemically stimulated by the juices of clam and is searching for food, a striking type of response already described. It continues for two minutes, and during the period the head is 2 cm. or more out of the tube.

9. Worm advances immediately and exhibits searching movements as in the preceding case. At some time it extends out of the tube 2 cm. or more, but at another time *draws back* into the tube, or to the end of it.

8. Worm moves forward beyond the mouth of the tube at once and shows searching movements as in 10 and 9. At all times during the 2-minute period, however, it is *less* than 2 cm. out of the tube, and it may during the time withdraw to the end of the tube, or into it.

7. Worm withdraws farther into tube. Before 2 minutes have passed, nevertheless, it moves forward to the end of the tube, or beyond the opening.

6. Worm attempts to grasp the end of the glass rod, or it may stretch forward several millimeters toward it, but afterwards it is motionless to the end of the period. No searching movements occur.

5. There is no visible reaction, positive nor negative.

4. Worm withdraws, but in less than 2 minutes moves forward. It does not progress, however, so far as the end of the tube.

3. Worm withdraws, but starts undulatory movements of the body (Fig. 1, *A*). By this activity not only is respiration aided but the animal's chemical environment is tested. If these movements are going on when the worm is touched they are continued.

2. Worm withdraws less than one centimeter and then *remains quiet*. Undulatory movements are *not* started.

1. Worm withdraws *more* than one centimeter, but *less* than two, and remains quiet.

0. Worm withdraws *more* than two centimeters and shows no further movement.

Although the difference between any two successive grades of reaction as outlined above is not great, forms of response which vary by two or three points are decidedly distinct, particularly to one familiar with the feeding behavior of *Nereis*.

On the completion of ten preliminary tests the results recorded for each worm were averaged and the first points of the three curves determined (Fig. 2, points I). The animal with the lowest average, or the one most negative to touch, was selected for immediate training. This proved to be worm *A*.

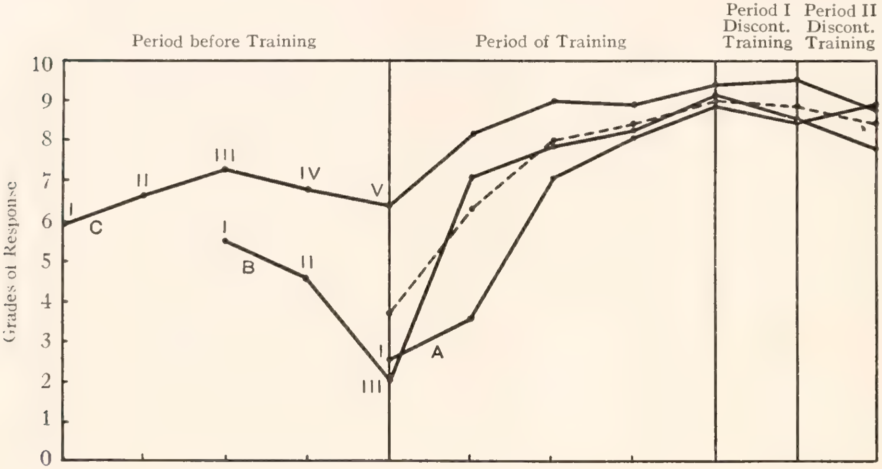

FIG. 2. Curves showing the changes in the reactions of worms to tactile stimuli alone (points I–V) and when followed by feeding (period of training). Their responses after two periods of interrupted training are indicated on the right. Each point on the curves represents an average of ten successive trials, and the dotted line shows the average of the three curves.

The procedure noted above was continued with worms *B* and *C*, the former for twenty more tests (determining points II and III of Fig. 2), and the latter for forty more (determining points II, III, IV, and V of Fig. 2), before their training periods were begun. During this time, therefore, these two worms served as controls. The same method of grading responses to tactile stimulation was used in the experiments to be described.

Period of Training

The training of worm *A* began on November 10, or two days after the completion of the first ten trials. During the period the same glass rod was employed in touching the worm and its care and manner of use were in no way modified. Again the behavior of the animal was noted for 2 minutes following tactile stimulation. At the end of that time, however, the worm *was fed*. This was accomplished by placing a small fragment of clam on the tip of another glass rod of approximately the same size and shape as the first. When the food was presented to the worm it was quickly seized and removed. If it happened that the head of the animal was inside the tube the end of the rod holding the clam was slowly inserted into the tube, when almost invariably juices emanating from the food stimulated the worm to move forward and take it. After using the rod it was washed in tap water and dried.

The worm was touched at least once a day, and usually twice, and was fed one piece of clam after each test. Thus the amount of food given it was about equal to that received before the training began and approximately the same as that taken by the controls. At the end of every ten trials an average grade of response was obtained which determined the points on the curve shown in Fig. 2.

After the completion of thirty[1] preliminary tests the training of worm *B* was started, worm *C* still being used as a control. It was not until December 26, when it had been given fifty tests with the glass rod unaccompanied by feeding that the training of worm *C* began. The same rods were used and the technique was unmodified.

The training periods of worms *A, B,* and *C* were closed November 30, December 23, and January 23 respectively, after each worm had been tested forty times.

The modification of the response to contact stimuli is shown by the curves in Fig. 2. From the beginning to the end of the training period there is a rise in the grades of the three worms varying from 3.0 in the case of worm *C* to 7.1 for worm *B*. On the other hand, before this period began the average of the last ten trials on worm *C* (Fig. 2, point V) shows a rise of only 0.5 when compared with the average of the first ten (Fig. 2, point I), whereas in the case of worm *B,* which was given thirty trials before training started, the third point on the curve (Fig. 2, point III) indicates a drop of 3.4.

Attention may be called to individual differences in the responses of the three worms to tactile stimulation. The averages of the first ten tests (Fig. 2, points I) led to the conclusion that worm *C* was least negative and worm *A* most so, and this order was still maintained at the close of the training periods. During this time, however, the three learning curves gradually converged until the range of variation in response was reduced from 3.3 grades to 0.5. It appears, therefore, that the differences in the reactions to touch existing at the start of the experiment were caused nearly, though not quite, to disappear.

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[1] Through error thirty-one tests were made. The last one was disregarded.

First Period of Discontinued Training

Having definitely modified the behavior of the three worms, it became of interest to determine how far the induced changes would be sustained over a period during which stimulation with the glass rod was discontinued. Accordingly, between the dates which marked the close of the preceding period and February 14, the worms were given four pieces of clam on alternate days. No independent tactile stimulation preceded the offering of food which was taken from the end of a glass rod. Contact occurred, therefore, only when the clam gently touched the head or mouth parts, in all probability about simultaneously with chemical stimulation. By February 14 worm *A* had been treated in this way for 75, worm *B* for 52, and worm *C* for 21 days.

Table I

Table showing grades of response of the three worms to tactile stimulation in successive trials at the beginning and end of the experiment

Worm	First Ten Trials *			Last Ten Trials of Training Period			Ten Trials at Close of First Period Discontinued Training			Ten Trials at Close of Second Period Discontinued Training		
	A	*B*	*C*	*A*	*B*	*C*	*A*	*B*	*C*	*A*	*B*	*C*
Trial 1.....	0	2	1	7	8	10	7	7	7	10	7	7
“ 2.....	1	6	6	10	8	9	4	10	10	10	7	8
“ 3.....	3	10	6	10	10	9	8	10	10	10	9	8
“ 4.....	0	5	10	10	10	10	7	8	10	10	8	10
“ 5.....	4	4	5	8	10	10	10	8	9	8	9	9
“ 6.....	6	0	6	10	10	9	10	6	10	8	7	9
“ 7.....	2	6	6	4	10	10	10	10	10	9	8	9
“ 8.....	3	6	5	10	8	10	10	8	10	8	8	10
“ 9.....	2	8	6	10	8	9	10	9	10	8	7	9
“ 10.....	5	8	8	10	10	8	9	10	10	8	8	9
Average	2.6	5.5	5.9	8.9	9.2	9.4	8.5	8.6	9.6	8.9	7.8	8.8

* The averages determined points I of graph.

In order to determine how much the modified behavior of the worms had changed during these intervals they were given ten tests by the method used in training them; namely, by first touching the animal with the rod and, at the end of 2 minutes, feeding it. The trials begun on February 14 were concluded February 20, when the results for each animal were averaged and the points plotted on the graph (Fig. 2).

It will be noted that the curves of two of the worms dropped only slightly; that of worm *B* 0.6 grades after 52 days without training, and worm *A* 0.4 grades after 75 days. On the other hand, the curve of worm *C*, after only 21 days without training, rose 0.2. An examination of Table I shows that in the case of worm *C* the average of the first five of the ten trials gave a response grade of 9.2 which is not above that (9.4) recorded at the close of the long period of training. The average of the last five tests, however, was 10. It appears, therefore, that this worm modified its behavior in the short series of ten trials, in truth, after the first, more than enough to offset any loss from 21 days lack of experience.

SECOND PERIOD OF DISCONTINUED TRAINING

After the trials reported above were completed the worms were not given further training for 35 days, although again they were fed four pieces of clam on alternate days. The manner of feeding, however, was modified in order to eliminate as far as possible any tactile stimulation. Instead of offering the clam from the end of a glass rod, by which method possibly the worm was occasionally touched by the food before it was recognized as such, the fragments were placed in the dish 2 or 3 cm. from that end of the tube toward which the posterior extremity of the animal was directed. Later, after turning about in the tube, and in most instances carrying on undulatory movements of the body, the worm received at first only chemical stimulation from the clam. It then advanced and seized the fragments.

The change in the worms' behavior at the close of this period was determined as before by giving them ten tests in which tactile stimulation by means of the glass rod was followed by feeding. The trials were begun March 26 and completed April 3, and the averaged reaction grades for each worm are indicated by the last points on the graph (Fig. 2).

As a result of this modification in the manner of feeding a sharper decline in the curves perhaps was to be expected, and the one representing the average of the three individuals does show this. Worm *A*, however, proved to be slightly more positive to tactile stimulation than it was before the training was discontinued for 35 days. It should be noted, nevertheless, that the curve of this worm is at no time higher than it was at the close of the training period.

A study of the table showing the results of individual tests on the three worms at the beginning and end of the experiment reveals not only a marked change from more negative to more positive reactions to tactile stimuli when associated with food, but also the persistence of this modified behavior. None of the forms of response below type seven involves food-searching movements (Fig. 1, *D, E, F,* and *G*), and twenty-five of the first thirty tests (ten on each individual) show an absence of such movements. At the end of the training period, however, only one test in thirty is graded below seven; in fact, only two below eight. At the close of the first period of interrupted training two tests of the thirty grade below seven, and at the end of the second period none at all. In view of the fact that the worms experienced no contact with the rod for over a month before the final thirty tests were made, the results indicate a decided retention of modified behavior.

Summary and Conclusions

1. *Nereis virens* when touched at the anterior end with a glass rod usually showed a reaction which was more negative than positive in character, whereas the response to chemical stimulation by materials derived from clams was decidedly positive.

2. When the tactile stimulus was followed by the presentation of fragments of clam three worms soon appeared to associate the two and learned to respond to touch in a more positive way. Finally after forty trials (for the most part given twice a day) the behavior of the worms following tactile stimulation alone could not be distinguished from that of worms which had been chemically stimulated by clam juices and were searching for food.

3. After periods of discontinued training the average of the three learning curves dropped slightly.

4. The results of this experiment are in accord with those of a previous one to which reference has been made (Copeland, 1930), where change in illumination was used as a sign for food, and indicate that *Nereis* readily forms a conditioned response.

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THE CHEMICAL NATURE OF THE PIGMENTS AND THE TRANSFORMATIONS RESPONSIBLE FOR COLOR CHANGES IN PALÆMONETES

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I. THE CHEMICAL NATURE OF THE PIGMENTS

The most prevalent pigment of *Palæmonetes vulgaris* is a reddish one which in the living shrimp varies in tint from a clear red to a brownish black, and in consistency from a homogeneous, viscous liquid to a granular gel. The yellow pigment, like the red, is always located within definite chromatophores. This pigment is a transparent, homogeneous liquid less viscous than the red. The yellow and red pigments, though most commonly present in the same chromatophore center, always retain their individuality as two distinct masses of pigment.

Lönnberg and Hellström (1932) studied spectroscopically the two pigments that they found in the lobster. In distribution between petroleum ether and methyl alcohol a yellow pigment remained in the petroleum ether (absorption bands in petroleum ether, 490 and 457 mμ) and a reddish pigment went into the alcohol layer (absorption band in ether, 494 mμ). Kuhn and Lederer (1933) have worked upon the brownish black pigment of the Norwegian lobster, *Astacus gammarus,* and have determined it to have the chemical formula $C_{27}H_{32}O_3$ and to this they gave the name astacin. The absorption band of astacin in pyridin was found to be very broad and to have a maximum at 500 mμ. These men also found in their extracts a small amount of a substance which they admitted was probably carotin. Later, Fabre and Lederer (1934) showed that astacin was present in a number of other crustaceans. Very recently Karrer and Loewe (1934) have concluded that astacin was a derivative of β carotin.

A study has been made of the absorption spectra of the pigments of *Palæmonetes.* Shrimps were boiled to convert the blue pigment to red, then dried and extracted with chloroform. The resulting extract was orange-red in color. It was quite photosensitive, bleaching to a pale yellow during a few hours exposure to sunlight. Figure 1 shows the absorption spectrum of the pigment freshly extracted in chloroform. There is a single asymmetrical band with a maximum at about 480 mμ.

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[1] Contribution No. 55.

This band is roughly the same shape as that determined by Redfield (1930), who examined a chloroform extract of the blood of the lobster, *Homarus*. The absorption spectrum of the lobster blood extract is also shown in Fig. 1.

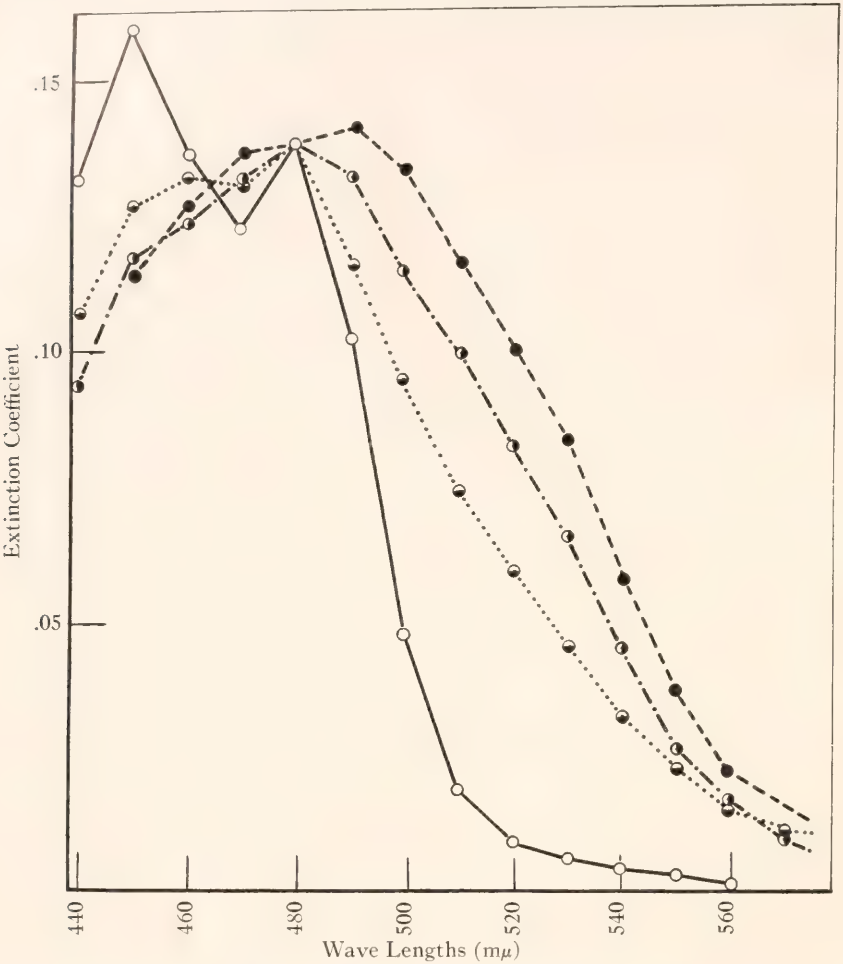

FIG. 1. The unbroken curve shows absorption spectrum of shrimp yellow pigment; ---- shows absorption band of extract of lobster blood (Redfield, 1930); ······ shows absorption spectrum of extract of shrimps which have little red pigment in chromatophores; and —·—·— shows the absorption band of extract of shrimp which have much red. All measurements are made of pigments in chloroform solution.

Kuhn and Lederer have found that when a petroleum ether solution of astacin is shaken with a solution of sodium hydroxide in 90 per cent methyl alcohol the astacin enters the methyl alcohol layer as a sodium salt of astacin. When this layer is acidified the astacin comes out of solution as violet crystals. It has been found that if the pigment obtained by extraction of the whole *Palæmonetes* with chloroform was dissolved in petroleum ether and then shaken with a solution of potassium hydroxide in 90 per cent methyl alcohol, a reddish pigment entered the methyl alcohol layer and a yellow pigment remained in the petroleum ether. Figure 2 shows the absorption spectrum of the petro-

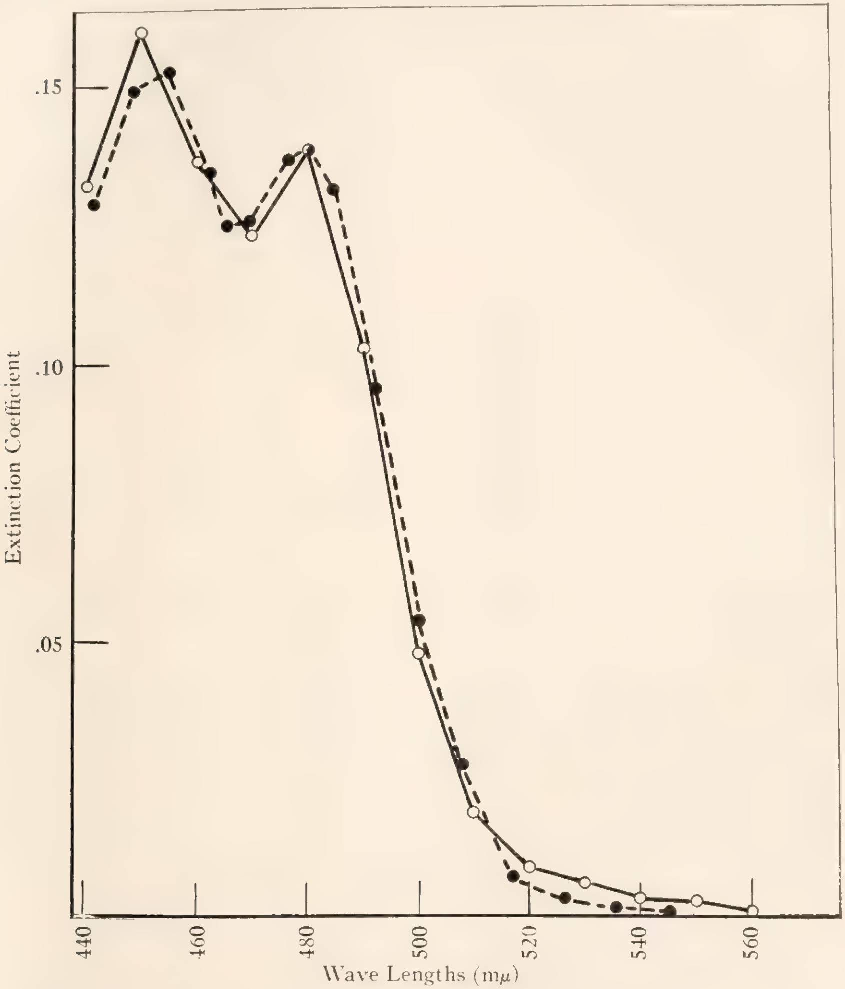

FIG. 2. The unbroken line represents the absorption bands of shrimp yellow pigment in chloroform; ---- shows the absorption bands of plant carotin in alcohol and ether as measured by McNicholas (1931).

leum ether component after it had been redissolved in chloroform. Plotted upon the same coördinates is the absorption spectrum of a solution of plant carotin in alcohol and ether as it was measured by McNicholas (1931). The shrimp pigment strikingly resembles plant carotin. Living *Palæmonetes* contain a greater proportion of yellow pigment than do lobsters and correspondingly a greater percentage of this fraction was obtained than was found by Kuhn and Lederer.

Samples of shrimp differing from one another in the relative proportions of red and yellow pigments visible in their chromatophores showed absorption spectra differing from one another in the extent to which the band was shifted in one direction or the other. The limits of this shift were defined by the absorption spectrum of carotin on the one hand and that of the lobster red pigment on the other. Figure 1 shows the absorption spectra of the whole pigment from two lots of shrimp. In one lot much red pigment was present in the chromatophores, and in the other there was considerably less. It thus seems probable that these differences are explained by the varying percentages of astacin and carotin in the extracts.

These results strongly suggest that the pigment of the yellow chromatophores of *Palæmonetes vulgaris* is carotin, $C_{40}H_{56}$, and that the pigment of the red chromatophores is one or another compound of astacin.

II. Chemical and Morphological Transformations Responsible for Color Changes

Thus far, nearly all the work upon color changes in crustaceans has been done upon the rapid responses of the chromatophore as indicated by the migration of pigment within the pigment cells. In reality, the well-adapted shrimps and prawns in nature owe their adaptations at least as much to the differential formation and destruction of pigments within the chromatophores as to the more rapid migration of the already formed pigments. Although this fact has been noted by the majority of workers in the field, little actual research has been devoted to it.

The following experiments were carried out upon adult *Palæmonetes vulgaris,* and were designed primarily to show the rate at which the pigmentation of the shrimps could be changed as a result of light, darkness, and black and white backgrounds.

Observations on Destruction of Red and Yellow Pigments

A shrimp was taken from a black background and placed upon white. In 20 minutes the red and yellow pigments had concentrated considerably. A blue network was left behind in the region that was formerly covered by the red and yellow pigments. The concentrated yellow was a clear and homogeneous pigment but the red contained much of the blue pigment mixed with it, making it purplish red and chocolate in color.

In about six hours the blue network had gradually faded and the yellow and brownish pigments had completely concentrated.

For the following six or eight days there were faint bluish processes extending from the brownish pigment. The blue pigment seemed to be diffusing away from this chromatophore and at the same time the chromatophore was becoming more and more reddish.

At the end of eleven days there was practically no blue pigment left in the red, but clustered around the red were many small blue triangular crystals.

After fifteen days the red pigment had become homogeneous and had decreased to only a small fraction of its original amount. Globules of red pigment had left the chromatophore and were in the tissues around it.

At the end of three weeks nothing was left of the red pigment except small droplets scattered about. The yellow chromatophore was still intact and functional but had lost a large quantity of its pigment. Observations were continued for two more weeks. The yellow pigment continued to decrease in amount but was still present at the end of this time.

Plate I, Fig. 7, shows a red, yellow, and blue chromatophore after two days upon a white background, and Fig. 8 shows the same region of the same shrimp after sixteen days upon the same background. Notice the small droplets of red pigment that still persist. The time required for the complete disappearance of the red and blue pigments is a function of the quantities present at the beginning of the experiment.

Observations on Formation of Red and Yellow Pigments

An experiment upon formation of pigment was begun by selecting a shrimp which had been kept upon a white background for five weeks and then seeking a chromatophore region where only yellow pigment remained. A photomicrograph (Fig. 9) was taken of the region and then the shrimp was placed in a salt water circulating apparatus upon a black background. At the end of five days the region was again examined. The yellow pigment was broadly dispersed and had assumed a pale orange tint. Some new red pigment had commenced to form. This last was surrounded by the characteristic blue network. The shrimp was placed upon a white background and after an hour the

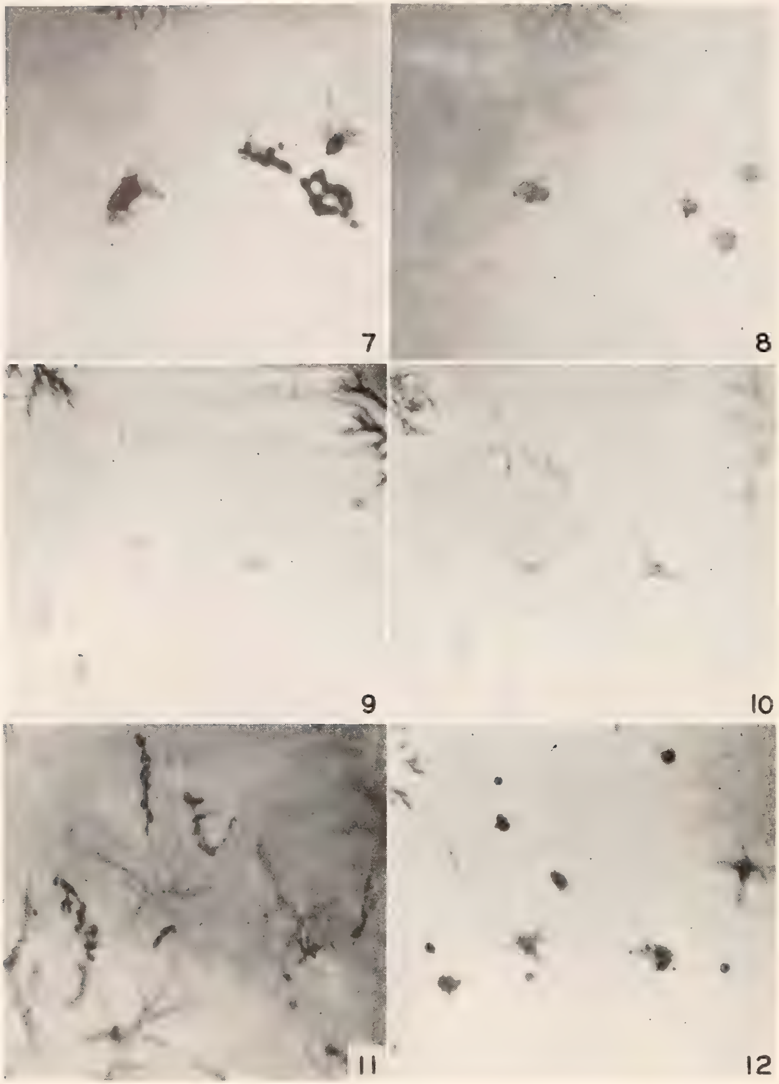

Plate I

Fig. 7. Red and yellow chromatophores of a shrimp which has been two days upon a white background.

Fig. 8. Same area as in the preceding figure but photographed after two weeks upon a white background.

Fig. 9. Some chromatophores in a shrimp that has been five weeks upon a white background. Only yellow pigment is present.

Fig. 10. Same area as in Fig. 9, and showing the formation of red pigment after five days upon a black background.

Fig. 11. Same area as in Fig. 10, but photographed after two weeks upon a black background. Much red pigment has formed and a blue network is visible.

Fig. 12. Same area as in Fig. 11, but the animal has been one day upon a white background.

region was again photographed (Fig. 10). After two weeks upon a black background the shrimp was becoming quite dark in color. Much red had formed and the blue network was heavy. After an hour upon white the chromatophores of the experimental region were photographed (Fig. 11). This was repeated after 24 hours upon white (Fig. 12) in order to determine definitely whether new chromatophore centers had actually been formed. It was unquestionable that new red and blue pigments had formed where there was not a visible trace of a chromatophore at the beginning. Thus it is reasonable to suppose that red and blue pigments may arise quite independently of the yellow. The original yellow pigment now had a reddish tint instead of its former lemon yellow.

The blue network which gradually faded out during the 24 hours upon the white background reappeared during the next three days upon a white background, but now, instead of being a continuous network the blue pigment appeared in clumps around the red masses of pigment. This blue remained for two weeks in spite of the white background.

In this experiment the red appeared before the blue and the latter seemed always associated with the former pigment rather than with the yellow.

Formation and Destruction of White Pigment

A shrimp that was kept upon a white background for nearly two months had such a quantity of white pigment in its chromatophores that when this was dispersed it covered a considerable portion of the body. Due to the transparency of *Palæmonetes* white shrimps were ordinarily quite well concealed even upon a black background. However, when the white pigment had increased in quantity to the extent that it had in this instance, the shrimp stood out in sharp contrast when placed in a black dish. The stomach, due to the mass of white chromatophores upon it and its muscular contractions, fairly sparkled. Even after this shrimp had been upon a black background long enough for the white pigment to concentrate, the shrimp was still studded with clearly visible white spots. This was not altogether due to the quantity of white pigment present, but also to the absence of the red and blue pigments which would ordinarily assist in concealing it in such circumstances. It is such an example as this that makes me disagree with those workers in the past who have asserted that the white chromatophores play no part in color adaptation.

I have made no record of changes within an individual shrimp, but the general tendency of more and larger white chromatophores when a shrimp was long upon a white background, and fewer and smaller white chromatophores in shrimps long upon a black background was striking enough to warrant this note of the fact.

Destruction of Blue Pigment

Although the origin of diffused blue pigment of *Palæmonetes* seemed to be the red pigment and the manner of its arrival in the tissues around the chromatophores and throughout the body seemed to be one of simple spreading, yet the disappearance of the pigment from the tissues appeared to be a question of destruction *in situ.* There has been no evidence that once the blue pigment was outside of the red pigment, there was any controlled migration back into it. An experiment was carried out to determine the effects of a white background and darkness upon the rate of destruction of the blue pigment.

Twenty-four deep blue shrimps were taken from a blue background and divided into two lots of twelve shrimps each. One of these lots was placed upon white background in the full light of a north window. The other lot was placed in total darkness. At the end of six hours the shrimps kept in the light had lost their blue coloration completely. In general appearance they were a yellowish white. The shrimps in darkness were promptly examined in a white light and found to be just as blue as at the moment when they were placed in the dark, but during the following six days there was a gradual disappearance of the blue network and the blue tint of all the tissues of the shrimps. Although blue was destroyed both in darkness and upon a white background in the light, the white background called forth a much more rapid destruction than did darkness.

A Quantitative Study of the Chemical Transformations in the Pigments

Hitherto no attempt has been made, as far as I am aware, to work out quantitatively the rates of pigment formation and destruction in crustaceans. A method is here set forth whereby quantitative determinations of the relative amounts of pigment in lots of shrimp subjected to various conditions can be made. The method is subject to some error due to the instability of the pigment itself, but since the results are relative and the technic uniform, it yields on the whole quite serviceable data. It gives one value which is the sum of the red, yellow, and blue pigments. The first step in the process is to convert the blue pigment to red by immersing the shrimps for a minute in boiling water. The work of Newbigin (1897) indicates that the red pigment that results from the heating of the blue is probably the same as the red that is present in the living shrimp.

The following is the technic that has been used in these experiments. Shrimps to be tested were dipped into boiling water for one to two minutes and then removed, cut into small pieces, and placed in a vacuum desiccator in darkness. At the end of 48 hours drying was completed and the dried shrimps were carefully weighed and then extracted with chloroform in the proportions of 30 cc. of chloroform to 1 gram dry weight of shrimps. The extraction was allowed to proceed for 24 hours, during which time the mixture was kept in a tightly stoppered test-tube in a refrigerator. After this interval of time the material was so well extracted that the remainder could be neglected. What would have been gained by a longer time of extraction would have been lost by the bleaching of the pigment. In the presence of light and air the bleaching occurred quite rapidly. Another source of error lay in the fact that the solvent was very volatile and thus the solution tended to concentrate in the course of the manipulations. Because of the volatility and photochemical activity of the material, the precautions taken during the drying and extracting were important ones.

At the end of 24 hours of extraction the solution was rapidly filtered and then the concentration of the pigment compared with that of a standard solution by the use of a colorimeter. Beer's law states that the amount of light transmitted by a solution varies inversely with the concentration of the absorbing substance. Hence a value for the concentration of the pigment in terms of the standard could be readily calculated. The standard solution was made by adding 1 cc. of methyl orange (.02 per cent in aqueous solution) to 16 cc. of an acetic acid sodium acetate buffer solution having a pH of 4.

One difficulty experienced in this method of obtaining values for the concentration was that different lots of shrimps contained different proportions of red and yellow pigments. This caused the solutions of pigment to differ from one another in tint. Considering that the colorimeter required the solutions compared to be the same color, some inaccuracy was introduced on this score. In order to determine the extent of this error several of the experiments were made and both the colorimeter and a spectrophotometer were used to measure the relative absorption of light by the solutions. The latter instrument was not affected by the color of the solution for here the light of only a definite wave-length was measured. The wave-length selected was 480 mμ, the peak of the absorption spectrum of the pigment of these crustaceans. The results obtained by the two instruments checked each other very well. For most of the experiments the colorimeter was used alone because of the rapidity of operation.

When the effect of a given set of conditions upon the amount of pigment in the shrimp was to be determined, a large number of shrimps were placed in the desired situation. At the beginning of the experiment and at three- or four-day intervals thereafter about twenty shrimps were taken from the lot as a sample to test for the concentration of the pigments. The value obtained was taken as a criterion of the concentration of pigment in the whole lot of shrimps at that particular moment. As this method rested to a great extent upon a statistical basis, much of the irregularity of the results was attributed to the fact that so few shrimps were taken in each sample. There is little doubt that had the samples consisted of as many as fifty shrimps, the data exhibiting the effects of the environment upon the pigment content of the shrimps would have more closely fit a smooth curve when plotted.

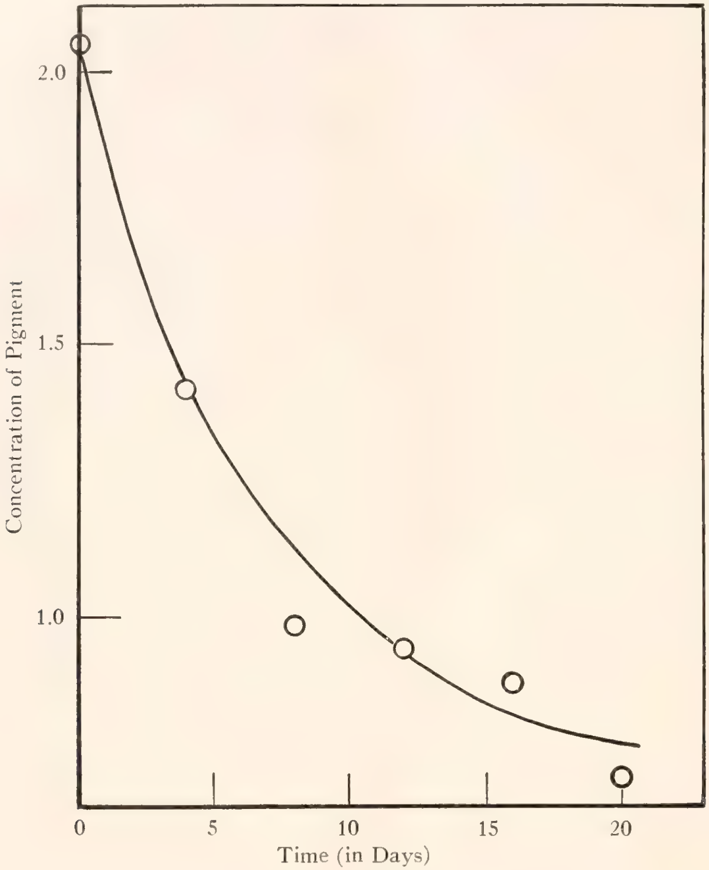

Fig. 3. Decrease in concentration of pigment in shrimps which were kept upon a white background in light. Concentration is stated in terms of a methyl orange standard solution.

Pigment Destruction upon a White Background

Approximately 250 shrimps were placed in a large white enamelled tub through which sea water was flowing. The tub was located in the center of a small room lighted by large north windows. During the course of the experiment the animals were fed upon finely chopped *Modiolus* and *Mytilus*. This experiment was repeated five times during the summer of 1932 with strikingly similar results. Figure 3 is typical of these experiments.

An experiment carried on in a similar manner except that the shrimps were starved over the period of twenty-four days yielded results showing the same rate of destruction of the pigments.

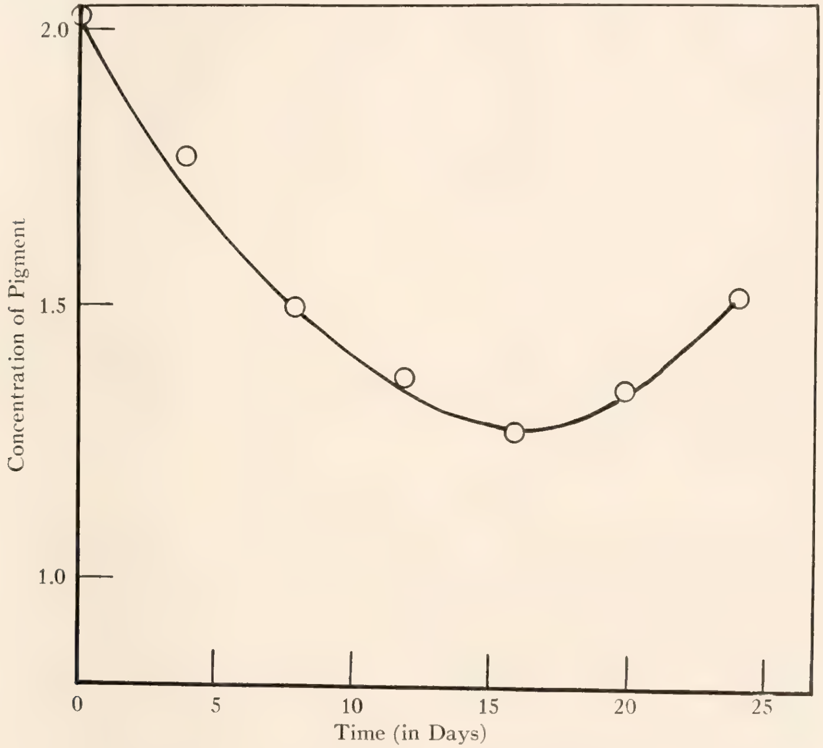

FIG. 4. Change in concentration of pigment in shrimps which were kept in darkness. Concentration is stated in terms of a methyl orange standard solution.

Effect of Darkness upon Pigment Quantity

In a second type of experiment shrimps were placed in large containers and allowed to remain in total darkness except for the brief moments when samples were being taken and the animals were being fed. At these times a weak red light was turned on. This experiment was repeated six times during the summer. Sometimes the shrimps were fed and at other times starved during the course of the experiment. Again there was no difference between the results obtained when the shrimps were fed and when they were starved. This can be explained by the cannibalistic nature of the shrimps. Figure 4 is typical of the results.

The change from pigment decrease to pigment increase at the end of about fourteen days was constant in all the experiments and was quite curious. It does not agree with the observations of Brooks and Herrick (1891). These men found that *Palæmonetes vulgaris* was bleached nearly white after eighteen days in darkness. Their animals were sealed up during the entire period whereas mine were subjected to the occasional stimulation of the red light. This question will bear further investigation as it perhaps holds an explanation as to why deep sea crustaceans are generally heavily pigmented with red. In the deep sea it is known that the conditions are those of nearly total darkness. On the other hand, crustaceans which inhabit underground caves where there is an absence of light are usually conspicuous by their lack of pigment.

Pigment Formation upon a Black Background

In a third experiment a batch of partially bleached shrimps was put into large crystallizing dishes the outsides of which were painted with flat black paint. The dishes were left in the daylight of the laboratory. Figure 5 gives an example of the type of result that was obtained in four different experiments.

Control Experiment

As a control experiment for the preceding ones, a large glass aquarium was filled with sea water and in the aquarium was placed an abundance of the alga, *Fucus,* from which these shrimps were taken in nature. As nearly as could be judged, this situation very closely simulated the normal habitat of the shrimps both in color of the environment and in light intensity. The results taken from shrimps kept in this aquarium are seen in Fig. 6.

Discussion of Results

From the graphs of the experiments just described the following facts are evident. Upon a white background in the light there is a very rapid destruction of pigment within the shrimps. This destruction is continued over the twenty-day period of the experiment. In another trial of the same nature the pigment was continuing to disappear even after thirty days. In this recorded case the pigment concentration decreased 65 per cent in the twenty days.

In darkness the rate of bleaching of the animal was considerably less than that seen upon a white background. This destruction of pigment continued at approximately the same rate for sixteen days and then there was a reversal to pigment formation. In various experiments the time of this reversal varied from eleven to sixteen days after the animals were placed in darkness.

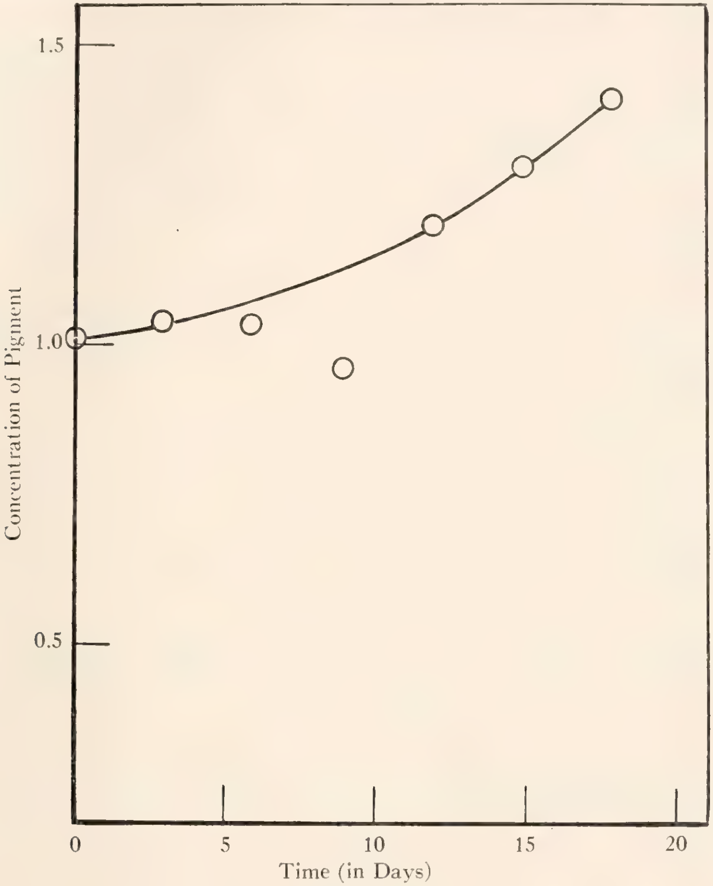

Fig. 5. Increase in concentration of pigment in shrimps which were kept upon a black background in light. Concentration is stated in terms of a methyl orange standard solution.

In the case of the partially bleached shrimps that were placed upon a black background there was at first only a slow formation of pigment and then an increase in rate. The rate continued to increase over the eighteen-day period of the experiment. During this time there was a 40 per cent increase in the amount of pigment in the shrimps.

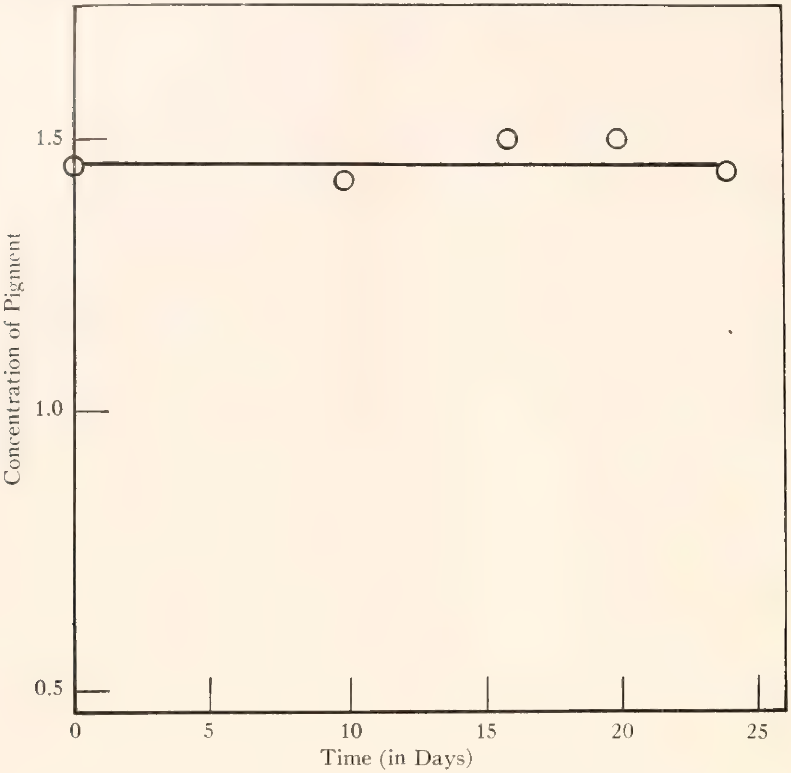

Fig. 6. Condition of concentration of pigment in shrimps which were kept in an environment which closely simulated the normal habitat. Concentration is in terms of a methyl orange standard solution.

The validity of this experimental technic was again checked in observing the behavior of the concentration of pigment of shrimps that were kept in apparently normal conditions. Here, over a period of twenty-four days, an interval of time as great as that of the longest experiment, there was neither an increase nor a decrease in the concentration of pigment.

It was shown by separating the extracted pigment of the animals into its two components by shaking a petroleum ether solution of the pigment with a strong solution of potassium hydroxide in 90 per cent methyl alcohol, that the shapes of these curves illustrating the rates of formation and destruction of pigment were almost wholly effected by the increase and decrease in the amounts of the red and blue. The rate of change of the quantity of the yellow pigment was relatively insignificant.

Controlling Mechanism of Pigment Formation and Destruction

Keeble and Gamble (1904), who were at the time working upon the prawn, *Hippolyte varians*, put forth an hypothesis to account for pigment formation and destruction. Since that time this hypothesis has been restated by other investigators such as Babák (1913), and Odiorne (1933). In substance it states that a dispersed pigment is increasing in amount while a concentrated one is decreasing.

The observations in this report confirm the hypothesis originated by Keeble and Gamble. For example, on a white background there is a strong concentration of the red and yellow pigments, an absence of diffusing blue, and a strong dispersion of the reflecting white pigment. Correspondingly there is an increase in the amount of white pigment and a decrease in the amounts of the other three pigments. A black background, just as the hypothesis calls for, brings about an increase in the quantities of the red, yellow, and blue pigments and at the same time a decrease in the amount of white pigment within the chromatophores. Darkness causes a destruction of red pigment at about half the rate seen in an animal upon a white background in the light and here the red pigment is slightly dispersed.

On the basis of these facts it is possible that pigment formation and destruction in adaptation to colored backgrounds may be controlled by the same humoral agents that are responsible for the control of migration of the pigments within the chromatophores.

I take this opportunity to acknowledge my indebtedness to Dr. G. H. Parker and Dr. A. C. Redfield for their invaluable criticisms and suggestions.

SUMMARY

The red pigment of *Palæmonetes vulgaris* appears to be astacin, while the yellow seems to be identical with plant carotin.

A method is set forth to measure the rate of formation and destruction of pigment within the shrimps. It is observed that the red and blue pigments are most rapidly formed and destroyed, while the yellow and white pigments are much more slowly changed.

Upon a white background in the light the red and blue pigments are destroyed very rapidly. Yellow disappears slowly.

Upon a black background in the light the red and blue pigments are formed.

Shrimps kept in darkness at first lose through destruction their red and blue pigment but after about two weeks there occurs a reversal of this process (pigment formation).

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HISTOLYSIS AND REGENERATION OF ANURAN TAIL SKIN[1]

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Introduction

Atrophy of the larval tail is perhaps the most readily observed phenomenon occurring during anuran metamorphosis. Descriptions of the progressive disappearance of this organ are usually limited, however, to the brief statement that the dorsal and ventral finny portions of the tail are the first to be resorbed, followed closely by the median region. Such a conception is entirely in accord with the atrophic changes in the tail of the salamander, for during the metamorphosis of this amphibian the dorsal and ventral tail-fins disappear while the median portion persists. The process, however, aside from its antero-posterior progression in the anurans, has received little if any critical study, with the result that the current view is based upon more or less cursory macroscopic observations.

The factors inducing the resorption of the larval anuran's tail, on the other hand, have received much more attention. Barfurth (1887) believed the atrophic changes to be due to a cutting off of the vascular supply to the tail, brought about by pressure of the growing urostyle upon the dorsal aorta. This idea was challenged by Bataillon (1891) and Mercier (1906). The former attributed the histolytic changes to a rearrangement of the capillary system in the tail, while the latter held that occlusion of the aorta by the urostyle was only partial. Morse (1918) and Bradley (1922), however, accepted Barfurth's suggestion as quite logical, and stated that such an occlusion, whether complete or partial, would result in the accumulation of CO_2 and various acid metabolites, causing an activation of the autolytic enzymes. Helff (1930) has recently demonstrated that removal of the urostyle anlage in larval forms does not alter in any particular the typical atrophy of the tail during metamorphosis. He pointed out that, such being the case, the urostyle could no longer be considered the fundamental factor inducing tail atrophy, and that the initiatory agent is probably of a more general nature. The investigations of Helff (1926), Lindeman (1929), Helff and Clausen (1929), and Clausen (1930, 1932) on the histolysis and regeneration of anuran skin and muscle transplantations all indicate that the initiation and maintenance of tail histolysis may be due to specific substances in the blood stream, or to a general lowering of the blood pH. Evidence in support of this latter factor has been advanced by the work of Helff (1932*a*) in which he found a definite drop in blood pH during larval involution.

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[1] This paper, together with Part II of this series of studies, was submitted to the Graduate School of New York University in partial fulfillment of the requirements for the degree of Doctor of Philosophy, April 1, 1934.

In the course of his studies on skin transplantation, Lindeman (1929) observed that tail-skin grafts, placed near the antero-posterior center of the larval tail, underwent an apparent migration anteriad during metamorphosis. Schubert (1926) noted the same phenomenon in the case of hind-limb buds transplanted to the tail. Such observations suggested that the anterior levels of the tail are more susceptible to the histolytic influence than the more posterior levels or, in other words, that the proximal regions of the tail are resorbed before the more distal. This possibility was investigated by Clausen (1930) and found to be the case. An antero-posterior gradient of susceptibility to histolysis was shown to hold for both tail-skin and muscle. In addition to supporting the contention that the histolytic factor is present in the blood, the findings of Clausen indicate that tail tissues possess a threshold value for the stimulus inducing atrophy which is specific for any given antero-posterior level.

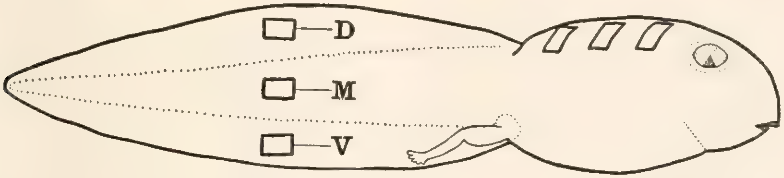
Spallanzani, as early as 1769, reported that the amount of tissue regenerated after amputation of the tadpole tail at different levels varies with the amount removed. Many later workers (Barfurth (1894), Morgan (1906), Ellis (1909), Durbin (1909), and Zeleny (1916)) have verified this observation, and, in more detailed analyses, found that the more anterior, or cephalic the level of injury, within certain limits, the greater the rate of regeneration. These writers seem to be in agreement that the differences in regenerative rate of tail tissue *in situ,* at different levels, are not due to specific qualities resident in the cells. Morgan (1906) proposed that a change in the pressure relationships of the part concerned is the stimulus for regeneration; division proceeding until intracellular and extracellular tensions come to equilibrium. Ellis (1908, 1909) accepted the above explanation as being consistent with experimental data, while Zeleny (1916) suggested that regeneration "must be under some central control, probably connected with general functional activity." More recently, Clausen (1930), in studying the regeneration of transplanted partially histolyzed skin from four levels of the antero-posterior axis of the tadpole tail, has approached this problem from another angle. His results established an antero-posterior gradient in regenerative rate for integument following partial histolysis, and indicated that this gradient in regenerative rate is due to factors inherent in the skin.

The purpose of the present investigation was threefold; first, to see whether or not a gradient of susceptibility to histolysis is present in integument from the dorso-ventral axis of the tadpole tail; second, to ascertain whether or not partially histolyzed skin, from different regions of the same axis, possesses a specific regenerative rate typical of integument for the level in question; and third, to determine the bearing of the results obtained on the mechanism of tail atrophy.

The writer is indebted to Professor O. M. Helff of this laboratory for suggesting the problem and for his direction during the course of the investigation.

Material and Methods

The stock used for all operations was large *Rana catesbiana* tadpoles measuring 105–115 mm. in length, with hind legs 4–8 mm. in length. The tadpoles were obtained from ponds in southern New Jersey and Long Island during 1932–33. They were kept in large aerated aquaria for a week or more in order to eliminate the less hardy specimens. All individuals selected for use were, insofar as could be determined by external indications, normal larvæ which remained unchanged under laboratory conditions.



Text Fig. A

The technique used in making the autotransplantations was as follows: After anesthetization in a 0.05 per cent aqueous solution of chloretone, the animal was placed on the stage of a dissecting microscope and rectangular pieces of integument, 3 by 5 mm. in size, accurately cut from the dorsal, median, and ventral regions of the tail at the level of its greatest dorso-ventral width (*D, M,* and *V*; text figure *A*). They were then carefully transplanted to areas on the back from which the skin had previously been removed (text figure *A*). The linear sequence of the grafts on the back was varied to obviate any possible effects due to the site of transplantation. Each animal was placed in an individual bowl immediately after the operation and allowed to remain 15 to 30 minutes without water. This tended to facilitate wound healing and prevent washing off of the transplant. At the end of this period the jar was filled with water and the specimen maintained under constant laboratory conditions for subsequent observations.

Following a healing period of seven days the grafts were measured under the dissecting microscope and the dimensions recorded. The areas computed from these measurements were considered as original areas of the grafts from which percentage reductions were later calculated. This procedure was necessary because the dimensions of the transplants changed slightly during the time allotted for healing. Those individuals in which one or more grafts failed to heal properly were discarded. The number of such cases, however, was quite small. Larval metamorphosis was induced at this time by feeding desiccated thyroid. Daily observations were made and the water changed as often as needed to keep it fresh. The grafts were measured, under the dissecting microscope, once each week during the early stages of involution and every third day in the later stages. At the arbitrary periods of 20, 50, and 80 per cent reduction in surface area, several of the transplants of each tail region were fixed in Bouin's fluid for sectioning. They were subsequently stained with Ehrlich's hematoxylin and counter-stained with eosin.

Results

Normal Tail Integument

The normal tail integument of *Rana catesbiana* does not differ materially from that of other larval anurans, and for this reason a detailed account of its structure is unnecessary. As an aid to the interpretation of the degenerative and regenerative processes, however, the main structural features will be reviewed briefly. Macroscopically, the tail-skin is translucent and of a very fine texture in contrast with the opaque, coarse nature of back-skin. With respect to pigmentation, the tail-skin contains more xantholeucophores and fewer melanophores than back-skin (Fig. 1).

Histologically, the integument can be resolved into three strata; the epidermis, the dermis, and the subcutis (*E, SC,* and *CT*; Fig. 5). The epidermis is composed of two or three layers of cells and an outer cuticular border. The cells of the outermost layer are low columnar to cuboidal in shape. The basal cells range in form from medium to tall columnar, and have within the cytoplasm fibrous, brush-like structures which are attached to the basement membrane (see *FS*; Fig. 5). Occasional mitotic figures as well as a few melanophores are present in this layer. One of the most striking histological differences between tail-skin and back-skin is found in the structure of the dermis. In tail-skin this consists of a single layer, the stratum compactum, but in back-skin there is in addition the stratum spongiosum, a region of loose connective tissue lying between the stratum compactum and epidermis. The stratum compactum of tail-skin is relatively thin and structurally quite homogeneous, whereas that of back-skin is formed by an upper layer of loosely arranged fibers and a denser more compact lower layer. Immediately beneath the dermis is the subcutis, consisting of loose connective tissue fibers and cells, lymphocytes, capillaries, and chromatophores.

The Degenerative Process

The sequence of degenerative changes in the integument is quite regular. Consequently, regardless of the source of the transplants, their histolytic changes are identical when the degree of resorption, as measured by the reduction in surface area, is the same. Therefore, the following description of the histolytic process is applicable to transplants from any one of the three regional sources used. The macroscopic changes, aside from a reduction in surface area, are confined to the pigmentation of the graft. When reduced 20 per cent the transplants are only slightly darker than normal skin (Fig. 2). They are considerably darker at 50 per cent reduction, with only a few or no xantholeucophores present (Fig. 3). When histolyzed 80 per cent they are of a uniform shade and very dark. In this later stage definite pigmentation spots may entirely disappear (Fig. 4). With continued degeneration the graft becomes smaller and darker in color, until finally it is completely resorbed and nothing remains but a dark scar indicating the line of fusion of the surrounding back-skin. To summarize: the depth of coloration is directly proportional to the degree of histolysis.

A detailed study of the progressive steps in the histological disintegration has not been attempted, but rather three arbitrary stages have been selected for microscopic examination, namely: when the transplants were 20, 50, and 80 per cent reduced in surface area. In the first stage the major changes are centered in the epidermis. This layer is thicker due to an increase of one or two cell layers, coupled with hypertrophy of the basal cells. The latter factor is in itself sufficient to account for the increased thickness in some cases (Fig. 6). The height of the outermost stratum of cells, as well as the cuticular border, is reduced about one-half as compared with the normal condition. Other signs of histolysis are seen in the fading of the fibrous, brush-like structures of the basal cells, and the invasion of lymphocytes into the epidermis. The dermis (stratum compactum) undergoes a slight reduction in thickness, exhibiting a few lymphocytes imbedded among the fibers. There is an increase in the cellular elements of the subcutaneous tissue; mainly lymphocytes but also a few eosinophilic leucocytes.

With the surface area reduced 50 per cent the histological structures have undergone extensive changes. The epidermis, while still consisting of two or three cell layers, is very greatly reduced (compare Figs. 6 and 7). The cuticular border of the now very much flattened cuticle cells has entirely disappeared. The fibrous, brush-like structures of the basal cells are no longer visible. In short, the epidermis has come to resemble, except for an occasional lymphocyte, the surrounding back-skin. Perhaps the most remarkable change is the almost complete resorption of the stratum compactum, which is represented by a very thin, loose, fibrous layer (*SC,* Fig. 7). The underlying layer of connective tissue contains a considerable number of blood cells, especially lymphocytes, but fewer than were present in the preceding stage.

When the transplant is reduced 80 per cent in surface area profound histological changes have occurred. It is very difficult to determine the structure of the epidermis in this stage, owing to the large number of melanophores aggregated in this layer (*ML,* Fig. 8). Furthermore, cellular boundaries are indistinct, and the nuclei vary widely in shape, size, and distribution. The cuticular stratum of cells is much thicker than in the foregoing stages, due to the inclusion of underlying cells from the intermediate layer. It contains one or two layers of flattened, ellipsoidal nuclei. Briefly, the epidermis presents a picture of great disorganization (*E,* Fig. 8). The original stratum compactum has disappeared, as well as much of the subcutis. The partial loss of the subcutaneous layer, it seems, results from the formation, underneath, of a thick stratum compactum derived from the stratum compactum of back-

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PLATE I

FIGS. 1–4. Sketches illustrating changes in the macroscopic appearance of autotransplanted tail-skin during metamorphosis. × 7.

FIG. 1. Autoplastic tail-skin graft seven days after transplantation to the back of a non-metamorphosing larva. No apparent histolysis.

FIG. 2. Macroscopic appearance of autotransplanted tail-skin, on the back of a metamorphosing larva, when reduced 20 per cent in surface area.

FIG. 3. Macroscopic appearance of autotransplanted tail-skin, on the back of a metamorphosing larva, when reduced 50 per cent in surface area. Note darkening of graft.

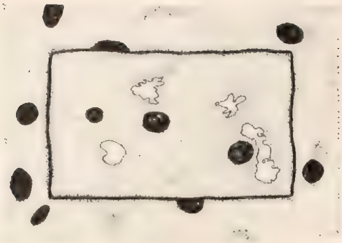
FIG. 4. Macroscopic appearance of autotransplanted tail-skin, on the back of a metamorphosing larva, when reduced 80 per cent in surface area. Uniformly very dark.

FIGS. 5–6. *CB,* cuticular border; *CL,* cuticle cell layer; *E,* epidermis; *FS,* fibrous brush-like structure; *SC,* stratum compactum; *CT,* subcutis; *ML,* melanophore; *FB,* fibroblast; *LM,* lymphocyte; *EL,* eosinophilic leucocyte; *ER,* erythrocyte.

FIG. 5. Section through normal tail-skin. × 510.

FIG. 6. Section through autoplastic tail-skin graft when reduced 20 per cent in surface area. The thickened epidermis, reduced cuticular border and cells are characteristic of this histolytic stage. An increase in number of lymphocytes and invasion by a few eosinophilic leucocytes are the principal changes in the subcutis. There is a slight reduction in thickness of the stratum compactum. × 510.

PLATE I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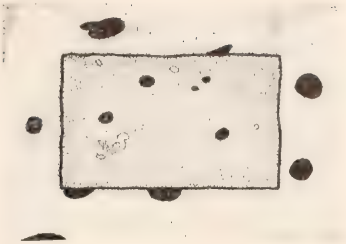
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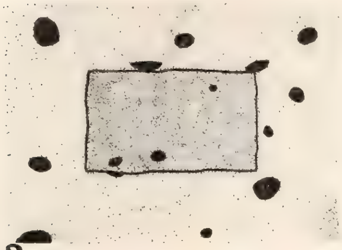
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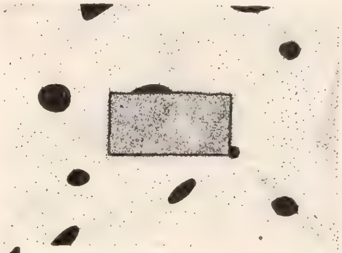
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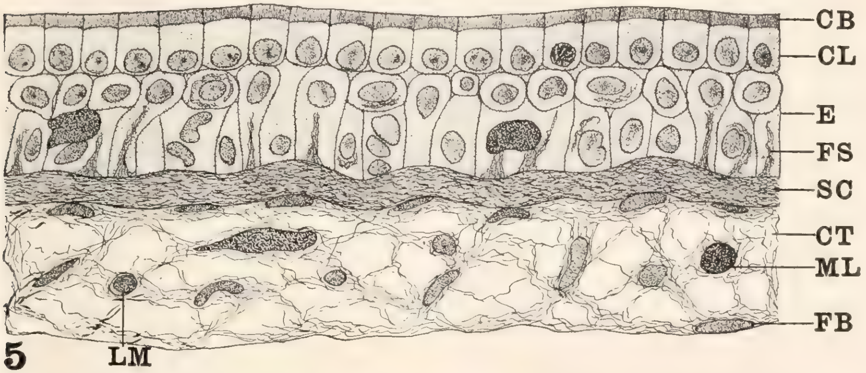
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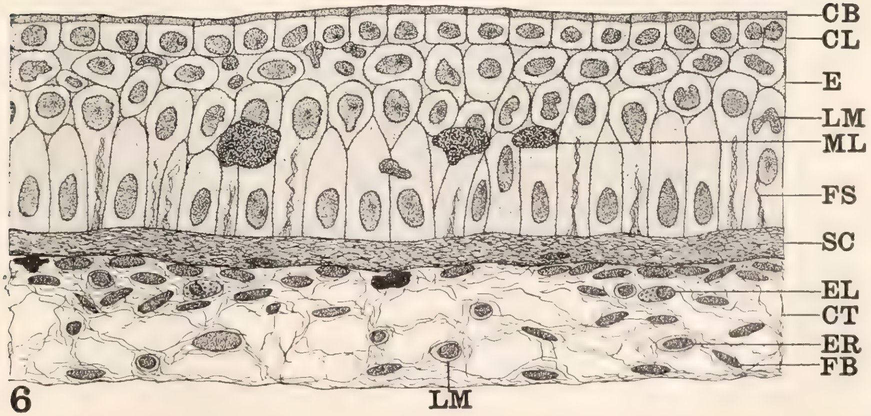
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skin. This newly formed stratum compactum is separated from the epidermis near the borders of the transplant by remnants of the subcutis, but in the central regions it is contiguous with the epidermis. It should be emphasized that this secondary stratum compactum does not possess the typical structure of that found in back integument. Although it is fully as thick as in the latter case, it is approximately of the same density at all levels, while back-skin compactum is composed of an upper loosely knit region and a lower more dense portion. Moreover, it is in intimate contact with the epidermis at some points, without the intervening stratum spongiosum so typical of back-skin.

Median-Ventral-Dorsal Sequence of Susceptibility to Histolysis

The basis for determining possible susceptibility gradients to histolysis in integument from the dorso-ventral axis of the tail was a comparison of the percentage reductions in the surface areas of the transplants. Supplemental histological examinations were also made. The case history of the grafts on one individual, from the time of transplantation until death of the animal, will illustrate the progression of histolysis on this basis (Table I). The letters *D, M,* and *V* of the table designate the regional source of the transplants, referring to dorsal, median, and ventral portions of the tail, respectively (see text fig. *A*). This table shows that histolysis is first evident in the median graft, next in the ventral, and finally in the dorsal. Furthermore, when this particular host animal died, *M* was completely histolyzed, *V* 80 per cent, and *D* only 56 per cent. It seems quite probable that the grafts would have disappeared, had the animal lived, in the order *M–V–D,* especially since that was the order of histolysis throughout the previous stages. The results do not warrant a definite conclusion on this matter, however, since in only one case did an animal live long enough for the complete histolysis of more than one graft. In this individual the ventral graft was the second one to disappear. Observations on the changing coloration of the transplants also corroborate the sequence of histolysis, *M–V–D.* The *M* graft was the first to become darkened, followed in order by *V* and *D.*

For a comparative study of histolysis in the various transplants the percentage reductions in surface areas were tabulated at four arbitrary periods; namely, when *M* was 20 per cent reduced, when *V* was reduced 20 per cent, when *D* was 20 per cent reduced, and when *M* was completely histolyzed or almost so. The results for each particular period were found to be quite uniform with only slight variations from the average. A summary of this extended tabulation is given in Table II and it will be noted that the histolytic sequence of the grafts at each of

TABLE I

Histolysis of tail-skin transplants. Case history DMV_{29}. Transplanted January 16, 1933.

Date of Measurements	Percentage Reduction in Surface Area		
	Regional Source of Grafts *		
	D	*M*	*V*
Feb. 9	0	16	0
Feb. 16	0	21	0
Feb. 25	0	40	8
Mar. 2	0	43	14
Mar. 7	6	47	20
Mar. 16	19	55	46
Mar. 22	23	55	48
Mar. 27	30	65	50
Apr. 7	42	80	66
Apr. 15	44	88	74
Apr. 17	56	100	80

* The letters *D*, *M*, and *V* refer to the dorso-ventral levels of the tail from which the transplants were obtained (See text fig. *A*).

these four stages, as indicated by the percentage reductions in surface area, is median-ventral-dorsal. Exceptions to this order of histolysis were noted in stage three (*D* reduced 20 per cent). At this time *V* was occasionally slightly more reduced than *M* and continued so for a short interval. However, in the earlier and later stages *M* was invariably histolyzed to a greater extent.

TABLE II

Comparative Histolysis of Tail-Skin Transplants

	Average Percentage Surface Area Reduction			
Regional source *	When M is reduced about 20% (183 cases)	When V is reduced about 20% (119 cases)	When D is reduced about 20% (50 cases)	When M is reduced 100% (10 cases)
D	0.7	4.36	20.8	68.0
M	21.9	46.9	49.4	100.0
V	1.67	20.9	48.3	84.0

* The letters *D*, *M*, and *V* refer to the dorso-ventral levels of the tail from which the transplants were obtained (See text fig. *A*).

Although the microscopic data are not as comprehensive as the macroscopic data, they are in close agreement with the results recorded in the tables. Consider, for example, the three grafts on one individual when *V* is reduced 20 per cent in surface area. The histological picture of *V* at this stage is shown in Fig. 6; *M* which is 50 per cent reduced is illustrated in Fig. 7; and *D*, having undergone only very slight reduction, shows little or no variation from normal integument (Fig. 5).

Regenerative Sequence of Partially Histolyzed Integument

In studying the rate or incidence of regeneration of partially histolyzed skin from the dorso-ventral axis of the tail, grafts reduced 20 and 50 per cent in surface area were secured according to the method described in the foregoing section of this paper. They were then transplanted homoioplastically to the backs of normal larvæ which would remain as such, under laboratory conditions, for at least six months. Approximately one hundred and eighty transplantations of this type were made. Beginning sixteen days after the second (homoioplastic) transplantation, four grafts of each of the three types (*D, M*. and *V*; 12 in all) were fixed at each successive three-day interval for histological examination. Although the degree of histological degeneration in grafts reduced 20 and 50 per cent in surface area had been determined in the previous experiments, additional cases were sectioned for controls at the time of homoioplastic transplantation.

The progression of regeneration, as with histolysis, is very regular, and, regardless of the source of the integument, the regenerative changes of any two grafts will be the same at any specific degree of regeneration. It will be necessary, then, to describe the process in integument from only one of the three source levels. Since regeneration is more extensive in skin histolyzed 50 per cent, in consequence of the greater histolytic disintegration, it has been selected as the type example (Fig. 7). It must be borne in mind that the chief aim of this investigation

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PLATE II

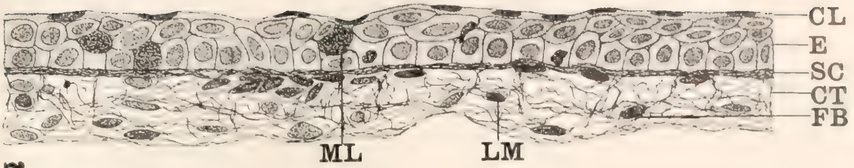
FIGS. 7–10. *CB*, cuticular border; *CL*, cuticle cell layer; *E*, epidermis; *FS*, fibrous brush-like structure; *SC*, stratum compactum; *CT*, subcutis; *ML*, melanophore; *FB*, fibroblast; *LM*, lymphocyte; *EL*, eosinophilic leucocyte; *ER*, erythrocyte.

FIG. 7. Section through autoplastic tail-skin graft when reduced 50 per cent in surface area. The cuticular border and fibrous, brush-like structures have disappeared. The cuticular cells and stratum compactum are greatly reduced. × 423.

FIG. 8. Section through autoplastic tail-skin graft when reduced 80 per cent in surface area. The epidermal layer is considerably disorganized and contains many melanophores. The stratum compactum is obliterated. × 510.

FIG. 9. Section through homioplastic graft 28 days after transplantation. Regeneration is manifest in the reëstablishment of the cuticular border, cuticle cells, and fibrous brush-like structures. The stratum compactum is being reformed. × 510.

FIG. 10. Section through homioplastic graft 37 days after transplantation. The cuticular border, cuticle cells, and epidermis in general are almost normal. Complete regeneration of the subcutis and stratum compactum requires about six more days. × 510.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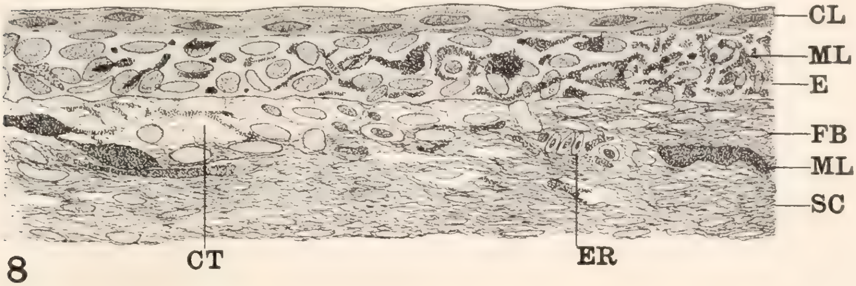
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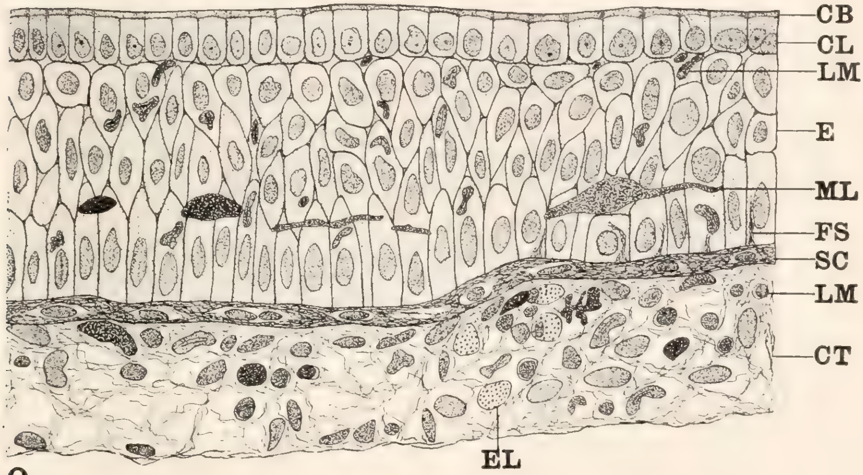
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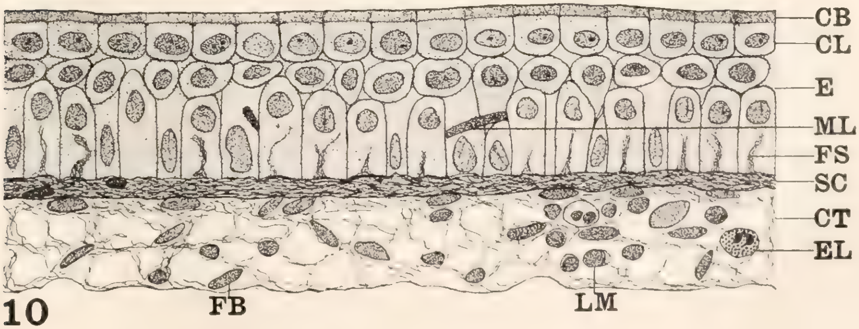
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was to determine the time necessary for complete reconstruction to occur, rather than to make a comprehensive study of the regenerative process.

The regenerative process begins a few days following homoiotransplantation. This is first indicated by an increased thickness of the epidermal layer. The exact mechanism of this increase has not been ascertained because the condition was well established prior to the first fixation period (16 days). However, since the area of the transplant is not appreciably reduced at this time, it seems unlikely that the epidermal thickening is due to a crowding or pressure effect of the surrounding back integument. After a regenerative period of 28 days the epidermal layer is several times thicker than it was at the time of transplantation, and has been invaded rather sparingly by lymphocytes (Fig. 9). A reëstablished cuticular border, although somewhat reduced, and the restored columnar form of the cuticular cells are characteristic of the epidermis at this stage. In the basal cells the fibrous, brush-like structures are being reformed. The stratum compactum has become prominent once more, containing lymphocytes and fibroblasts imbedded among its fibers. The subcutis contains several lymphocytes and a few eosinophilic leucocytes. Other blood elements are quite probably present but the staining technique used was inadequate for a critical determination of these cells. With continued regeneration the component parts of the epidermis gradually return to normal; first the cuticular border and cuticle cells, then the intermediate and basal layer of cells, and finally the fibrous, brush-like structures of the basal cells. Meanwhile, the fibers of the stratum compactum become more firmly bound together, with very few cells among them. The cellular elements of the subcutaneous tissue decrease in number so that, except for a few more lymphocytes and an occasional eosinophil, it closely resembles the normal subcutis. Figure 10 shows such an intermediate stage in which the epidermal layer is almost normal, with the stratum compactum and subcutis rapidly nearing complete reconstitution. Further growth of the stratum compactum and diminution in the number of lymphocytes in the subcutis, accompanied by the disappearance of the leucocytes, restores the original normal structure of the integument (Fig. 5).

The appearance of the homoiotransplants undergoes very little alteration during the greater part of the regenerative period. Usually within six to nine days before complete regeneration, however, the normal coloration returns, although the grafts may sometimes become lighter in color at an earlier stage. This rather sudden change in depth of coloration near the close of the regenerative process is probably correlated with the withdrawal of cellular elements (lymphocytes and leucocytes) from the subcutis and a decrease in the number of cell layers in the epidermis. A re-arrangement of the melanophores is also a contributing factor. Another point of considerable interest is that at no time after a transplant had properly healed was it replaced by the surrounding back-skin.

TABLE III

Regeneration of Tail-Skin Transplants

Regional Source *	Initial Reduction in Surface Area	Days Taken for Return of Normal Histolytic Picture				Average Time
		Case 1	Case 2	Case 3	Case 4	
	per cent					
D	20	35	37	37	40	37
	50	49	52	52	49	50
M	20	28	31	31	31	30
	50	40	43	43	40	41.5
V	20	31	34	34	37	34
	50	43	46	46	49	46

* The letters *D*, *M*, and *V* refer to the dorso-ventral levels of the tail from which the transplants were obtained (See text fig. *A*).

Histological examinations revealed that complete reconstruction, in both 20 and 50 per cent histolyzed grafts, occurs in the median transplant before it does in either the ventral or dorsal, and in the ventral before the dorsal. That is, the three different regional grafts do not achieve complete regeneration at the same time but in the order median-ventral-dorsal. These results are set forth in Table III. The data in this table give only the results for complete reconstitution, but it should be borne in mind that this same sequence holds for any intermediate stage. In other words, *M* is the first graft to attain any given stage of regeneration, succeeded in order by *V* and *D*. Thus, partially histolyzed integument from the dorso-ventral axis of the tadpole tail possesses a sequence either in the time of regenerative incipience or the rate of regeneration which is of the same order as the histolytic sequence, namely, median-ventral-dorsal.

DISCUSSION

The results are clear in that integumentary grafts from different dorso-ventral levels of the tail do not histolyze simultaneously but in the sequence median-ventral-dorsal. The cause of this histolytic sequence is not entirely clear; it may be due to a time or rate differential, or a combination of these two factors. In the initial stages, however, it is unquestionably the former, since signs of histolysis are first evident in the median graft, next the ventral, and finally the dorsal. This order of histolytic incipience was true for all but nine of the 183 cases studied. In these exceptions the only change was a reversal in the order of appearance of histolysis in the ventral and dorsal grafts. It may be that in the later stages of histolysis a rate differential becomes a factor in the maintenance of this sequence. Since the histolytic rate of each graft is characterized by alternating periods of acceleration and deceleration, it is difficult to ascertain possible rate differences. In fact, rate changes in any one graft appear to be roughly equal to those occurring in other regional grafts. For this reason the writer is inclined to believe that the described sequence is largely the result of a difference in the time of histolytic incipience and not due to differential rates.

Since grafts from different dorso-ventral regions of the tail complete histological regeneration in the order median-ventral-dorsal, it is obvious that tail-skin possesses a regenerative as well as an histolytic sequence. The relative time required for each regional graft to restore its normal histological structure was the basis used in determining this sequence. An exceptionally large number of homoiotransplantations were necessary in making this determination because of the comparatively long regenerative period of partially histolyzed integument. In fact, sixty 50 per cent histolyzed grafts, each involving two transplantation procedures, were required to determine the time necessary for complete histological reconstitution of the median transplant alone. Consequently, the grafts were not fixed, for sectioning and subsequent examination, in the earlier regenerative stages. The first fixation period, for the most part, was sixteen days after the second (homoioplastic) transplantation, at which time regeneration had begun in all three regional grafts. For this reason the question of whether or not the sequence is due to a difference in the time of regenerative incipience, rate, or a combination of these factors cannot be answered without a more detailed study. The histological sections of such early stages as were made, however, indicate that the time of regenerative incipience is not the same for each regional graft. In view of this it may not be entirely out of place to suggest that further work may show the regenerative differential to be dependent largely, if not entirely, upon differences in the time of regenerative incipience.

In the regenerative series of experiments no incompatibility between homoiotransplants and host could be detected after the grafts had healed.

There were a few cases in which the grafts failed to "take," but in most cases this was probably due to errors in transplantation technique. The pigmentation and general appearance of the homoioplastic transplants were at all times characteristically those of tail-skin. Herrick (1932), on the other hand, reported that homoiotransplants of side and belly-skin acquire typical back-skin pigmentation, and hence concluded that homoioplastic grafts are replaced by host tissues. He failed to find any evidence, however, for an overgrowth or undergrowth of the transplant by host epidermis as suggested by Cole (1922). In the present work there were no indications of an invasion of the graft by host epidermis. Furthermore, the stratum compactum seems to be restored by new formation in and under the old graft layer, a condition previously noted by Helff (1926). Moreover, the regenerated stratum compactum is of the typical tail-skin type even though surrounded by the distinctly different back-skin compactum. It is concluded, therefore, that in the present experiments an actual regeneration of the transplanted integument occurred, which did not involve replacement by host tissues.

The fibrous, brush-like structures of the epidermal cells agree in their morphology, and in their reactions to degenerative and regenerative stimuli, with the "coarse mitochondrial threads" described by Speidel (1926). Since the technique used did not permit of a critical cytological study of these elements, there is no evidence either for or against Saguchi's (1913) theory that they are fused mitochondria. Their principal interest in the present investigation lies in their characteristic behavior during the regeneration and degeneration of the integument.

Granting that histolytic and regenerative sequences do exist in tail integument, it is interesting to inquire as to their physiological basis. Since tail-skin after transplantation to the back histolyzes during metamorphosis, the stimulus inducing atrophy must be present in the general circulation, a condition long advocated by Helff et al. The histolytic sequence of grafts from different dorso-ventral levels in the order median-ventral-dorsal, then, can only be due to inherent differences in the skin, for each graft is subjected to the same atrophic stimuli. Likewise, the restitutional phenomena occurring in partially histolyzed tail-skin grafts must also be dependent upon inherent qualities of the integument. Such grafts, when removed from metamorphosing larvæ and transplanted to the backs of non-metamorphosing tadpoles, complete regeneration in the sequence median-ventral-dorsal. This sequence of regeneration cannot be due to differences in environmental conditions for all grafts were acted upon by the same external forces, the result of a common transplantation site. The logical interpretation is that tail integument possesses regenerative potentialities which differ for each dorso-ventral level. Any statement as to the basis for these inherent potentialities is certain to be somewhat speculative, but Helff's (1932*b*) recent demonstration that the anterior levels of the tadpole tail are electro-negative to the more posterior regions suggests a possible explanation. Although it is generally agreed that potential differences arise as a result of differential metabolic rates, there is considerable disagreement as to whether the region of higher rate is electro-negative or electro-positive to the region of lower rate. However this may be, the important point is that there is a difference in potential between the cephalic and caudal regions of the tadpole tail which, according to the above interpretation, in turn indicates the existence of a metabolic gradient. On this basis the writer suggests, tentatively, that the inherent histolytic and regenerative potentialities of tail integument, as evidenced by the present work, are determined or conditioned by differences in metabolic rate characteristic of the three dorso-ventral tail regions studied.

SUMMARY

1. Uniform-sized tail-skin grafts were secured from extreme dorsal, ventral, and one intermediate region of the tail and transplanted, autoplastically, to the backs of non-metamorphosing *Rana catesbiana* tadpoles. Artificial metamorphosis was then induced and the histolysis or reduction of the grafts studied. The median-source graft was invariably the first to undergo reduction followed in order by the ventral and dorsal, regardless of their antero-posterior transplantation sequence on the back.

2. Similar grafts of tail-skin were transplanted, autoplastically, to the backs of non-metamorphosing larvæ, and artificial metamorphosis subsequently induced. At arbitrary stages of reduction the grafts were retransplanted, homoioplastically, to the backs of non-metamorphosing larvæ and allowed to regenerate. The median graft was the first to complete regeneration, as determined by histological reconstitution; the ventral being the second and the dorsal third.

3. The histolysis and regeneration of tail-skin always involves an orderly sequence of cellular changes. For both of these processes the sequence was determined as epidermis-dermis-subcutis.

4. It is concluded that the histolytic sequence is due largely to a time differential in the onset of histolysis. The regenerative sequence is probably the result of a time differential in regenerative incipience.

5. The physiological basis of these sequences is inherent in the tissues, and it is inferred that they may be determined or conditioned by different metabolic rates typical of each particular level.

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THE MOVEMENT AND REGULATION OF CILIA ON THE PARAPODIA OF NEPHTHYS BUCERA, EHLERS [1]

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The mechanism concerned with ciliary control has been studied extensively, from both the morphological and physiological features of this effector system. Included in the physiological aspects of these studies are such phases as ciliary movement and ciliary coördination. Even though numerous publications concerned with cilia have appeared, we are still uncertain as to the exact mechanism responsible for ciliary movement and of the principle involved in ciliary coördination. Perhaps the smallness of cilia and the fact that they break up so readily during either chemical or mechanical treatment are problems preventing a thorough understanding of this type of effector system.

The cilia on the body wall and on the branchiae of *Nephthys bucera* have been used in the experiments reported here because their size is unusually large and they appear on structures in convenient locations for study. Since *Nephthys bucera* has not been studied in detail, even by Ehlers (1868), who described the type specimen and who made no mention of any ciliary organs, a part of this report deals with the structure of these cilia-bearing regions. Consideration also is given here to the problem of ciliary coördination.

The distribution and function of cilia on the parapodia of *Nephthys bucera,* together with the direction of their effective and metachronal waves, have been described by Coonfield (1931). These cilia are located on the branchia of each notopodium and they continue in a single line on the body wall to the base of the neuropodium. They are definitely grouped into small tufts, each of which is limited to a single cell (Figs. 5, 7, and 8). There are 18 or more of these tufts on the outer and on the inner margin of each branchia and 20 or more on the body wall between the two parts of a parapodium. Each ciliary tuft is made up of 40 to 50 large compound cilia that vary in length, the shorter ones being at the margins of each tuft. Also the tufts decrease in length gradually from the longest ones at the base of the branchia to the shortest one on its tip. The cilia on the body wall are slightly longer

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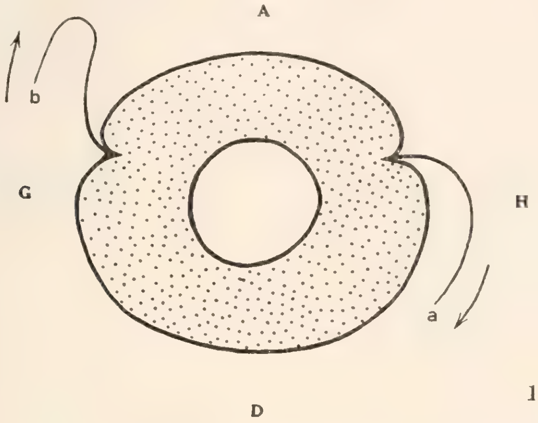
[1] Contribution No. 14 from the Department of Biology, Brooklyn College. This work was begun at the Zoölogical Laboratories, Harvard University.

than those on the branchiae. The longest cilia measured were about 62μ while the shortest ones were 5.6μ. Gray (1928) has stated that cilia vary from 0.1μ–0.3μ in diameter and are often 15μ in length. This author is obviously including only the simple type of cilia, for Carter (1924) and Bhatia (1926) have given 30μ for the length of the complex latero-frontal cilia of *Mytilus*. That these large cilia of *Nephthys* are of the compound type is shown definitely by their breaking up into smaller units when treated with fixing reagents and when a ciliated cell is disintegrating after having been punctured. This breaking up into smaller units begins at the tip of the cilium and progresses to its base. In that these cilia are compound, they are similar to those of the nudibranch veliger (Carter, 1926) and to the latero-frontal cilia of *Mytilus* (Carter, 1924). Although the ciliary units could be seen, it was not possible to determine their exact form.

According to Gray (1928), there are three types of ciliary movement: the pendular type which includes only those cilia that vibrate backwards and forwards by flexure at their bases; the flexural type composed only of cilia that move by a flexure beginning at their tips and passing down to their bases, thereby bending the cilia into hook-like structures; and the undulatory type which is characterized by a series of waves passing from the base of the cilium to its tip. The cilia of *Nephthys bucera* move according to the flexural type and thus resemble those of the gills of *Mytilus*. The cilia of *Mytilus* (Gray, 1920) are stiff in their effective beat and are limp in their return stroke. The cilia of *Nephthys* are not quite as flexible in their recovery beat as are those of *Mytilus* and they come to rest at the end of the effective beat rather than at its beginning.

The metachronal wave of the cilia of *Nephthys* as it moves from the base of the outer edge of the branchia on around the tip and along the body wall shows clearly a rhythmic movement. The metachronism is produced by simultaneous beating of the cilia at corresponding edges of each tuft, and thus moves along the line of these tufts in a perfectly synchronized pattern. This wave moves in a clockwise direction, passing towards the neuropodium on the right side and away from the neuropodium on the left side of the animal (Coonfield, 1931). It is interesting to note that this direction of ciliary movement corresponds to that of the prototrochal cilia of *Spirobranchus tricornis* and *Eunice fucata* as reported by Mayer (1910). Since the direction of the effective stroke and that of the metachronal wave of cilia on animals are in most cases either the same or directly opposite to each other, it is of further interest to note that these directions of movement in *Nephthys* are at right angles to each other. The cilia of this worm at corresponding regions on the opposite margins of a branchia reach the opposing phases of their movement at the same time. Thus when a cilium on one side of the branchia reaches the end of its effective stroke, the corresponding one opposite is at the end of the recovery stroke (Fig. 1). This form of beating produces a current which carries suspended particles in a circular fashion around an isolated branchia. The effect of ciliary movement on the body wall, however, combines with that of the branchial cilia to carry liquids and other materials posteriorly along the body of the worm. This movement of water over the branchiae of *Nephthys* is a definite aid to respiration. This is of significance since this animal usually lives in about six inches of sand and does not possess any other accessory respiratory organs.



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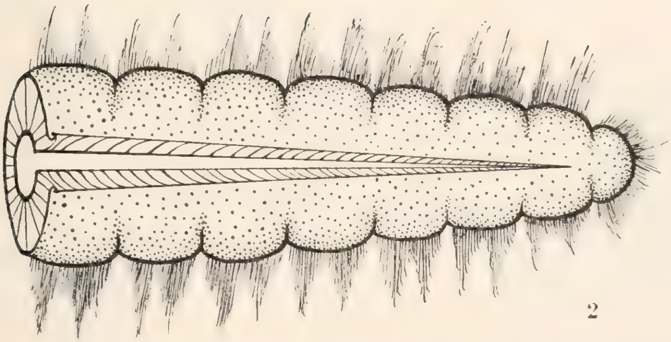

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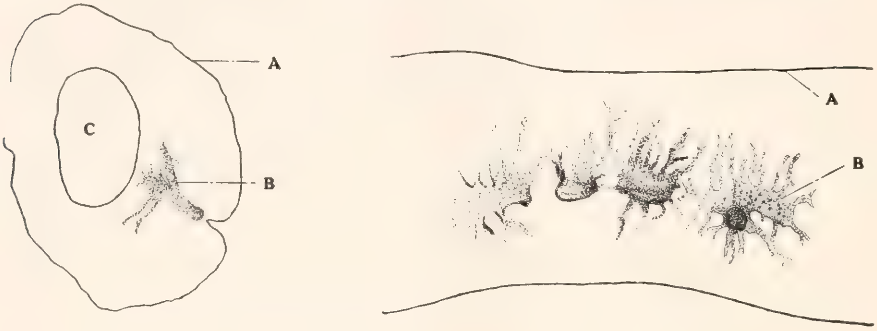
FIG. 1. Diagrammatic cross-section of a branchia, showing the location of its cilia with the relationship of their phases of movement. *A,* anterior; *D,* posterior; *G,* inner margin; *H,* outer margin; *a,* end of effective beat; *b,* end of recovery stroke.

FIG. 2. Diagrammatic view of a branchia, showing the cut made in testing for the ciliary coördinating system.

Structures in the Ciliated Region as Shown by Stains

Iron hematoxylin, with or without a counter stain, showed a tuft of cilia to be limited to a single cell. Each of these cells possesses a single nucleus. This condition is quite unlike the syncytial form as reported for the latero-frontal epithelium of *Lampsilis* by Grave and Schmitt (1925). These ciliated cells of *Nephthys* are distinctly separate. Small intracellular fibrils which pass from the base of each cell to its cilia could be seen (Figs. 7 and 8). There was no evidence of a cone-shaped fibrous structure as found by Grave and Schmitt (1925) in the latero-frontal epithelium of the gill of *Lampsilis* nor was there any degree of association between these fibers and the cell nucleus. Iron hematoxylin did not show any basal corpuscles associated with the cilia, as reported by Saguchi (1917) for ciliated epithelium of *Anodonta*. Many fibers were shown by this stain within the basal membrane beneath these ciliated cells. There was no definite morphological association, however, between these fibers and those of the cilia even though these fibers were near each other. Vom Rath's stain showed these cells as having a granular cytoplasm rather than a fibrous one as was shown by iron hematoxylin (Fig. 9). Immersion of pieces of a branchia or even whole segments of *Nephthys* in very dilute solutions of methylene blue from 1 to 8 hours produced effects on the cytoplasm of the ciliated cells similar to that of Vom Rath's stain, except that in methylene blue the cytoplasm stained more deeply in the central part of the cell near the nucleus and showed the root-like substance converging toward the nucleus (Figs. 3, 4 and 6). If, however, the tissue remained in methylene blue 8 hours or longer the stained substance was shown broken up near the basal membrane (Fig. 6). Neither basal corpuscles nor ciliary rootlets were shown by methylene blue.



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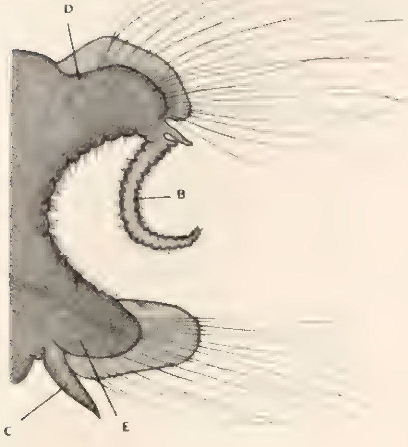
Figs. 3 and 4. Camera-lucida drawings of ciliary regions to show the effect of methylene blue on ciliated cells. The drawings are of the cross-section and sagittal section respectively. *a,* branchial wall; *b,* intracellular stained materials; *c,* branchial cavity. (Methylene blue $1\frac{1}{2}$ hours, 7 μ, $\times$ 440.)



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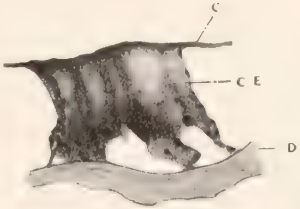

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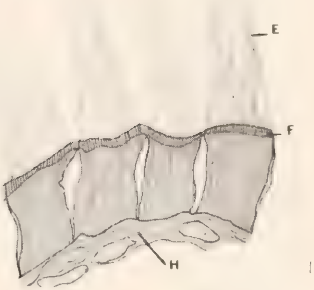

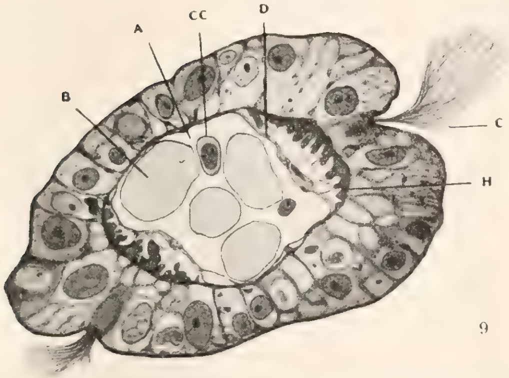

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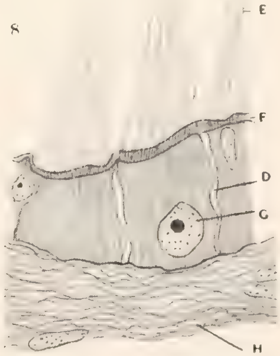

FIG. 5. Camera-lucida drawing of a parapodium of the right side of *Nephthys*. *A*, setæ; *B*, branchia; *C*, ventral cirrus; *D*, notopodium; *E*, neuropodium.

FIG. 6. Camera-lucida drawing of a ciliated cell of *Nephthys* to show the effect of staining with methylene blue at 8 hours. *C*, cuticula; *CE*, cell structure; *D*, basal membrane. (7 μ, × 900.)

FIGS. 7 and 8. Camera-lucida drawings of ciliary regions to show intracellular materials stained in iron hematoxylin, just to one side of the nucleus and through the nucleus respectively. *D*, intercellular space; *E*, cilia; *F*, cuticula; *G*, nucleus; *H*, basal membrane. (10 μ, × 440.)

FIG. 9. Camera-lucida drawing of a cross-section of a branchia, showing the structure of this organ; *A*, branchial cavity; *B*, blood vessel; *C*, cilia broken into their units; *CC*, cœlomic corpuscle; *D*, peritoneum; and *H*, branchial muscles. (Vom Rath, 6 μ, × 900.)

Reactions of Cilia to Physiological Tests

The ciliated regions of *Nephthys* are in convenient locations for employing physiological methods in testing for the ciliary regulatory system. Either pieces of a branchia or a whole branchia isolated from an animal's body will live for several hours without either the rhythm or the amplitude of their cilia being changed from the normal. A single isolated branchia was slit from end to end on one side between the opposite rows of cilia (Fig. 2). The beating of its cilia with their metachronal wave continued in the regular manner after this cut was made. Within a few hours, however, this tissue disintegrated, but the ciliary beating was normal until the beginning of disintegration.

Pieces of branchiae were impaled on a cover slip by means of a micro-needle and the ciliated cells were treated in the following ways: 1. A single ciliated cell was punctured with a micro-needle and it broke up immediately. Its cilia stopped beating as soon as this dissipation reached them and thus confirmed the views of Chambers (1924). 2. A cell bearing active cilia, intact with other similar cells, was pressed near its base. Its cilia became quiescent as this pressure was applied, but the cilia of the adjoining cells continued their regular beating. The quiescent cilia regained their beating and rhythm upon the release of this pressure. 3. A single active ciliary cell between two other similar ones was destroyed by the puncture method. The cilia on this cell ceased beating while those on the other cells did not change their rate or their metachronal movement. 4. Pressure was applied on non-ciliated cells adjoining active ciliated ones without affecting these active cilia. 5. One ciliated cell was pressed at the bases of its cilia near the center of the tuft. The cilia in the pressed region stopped beating. Those on either side of the quiescent cilia continued to beat, but with a slower rate. As soon as the pressure was released, however, all of the cilia resumed their normal beating. 6. A group of ciliated cells was stretched by a pull with microneedles. Their cilia became quiescent, but resumed normal movement as soon as the stretching was discontinued. 7. A few cilia in a single tuft were held for a few seconds. The other cilia in this tuft continued to beat without changing either their rate or their metachronism.

Discussion

The beating of cilia serves the animals on which they are located in one of two specific ways. In the one, they beat continuously in an automatic manner and move substances along a surface. Though the activity of these cilia is thought by some to be independent of other tissues and therefore not controlled by the nervous system, evidence has been presented by Agersborg (1923), Albertoni (1891), McDonald (1927), Merton (1923), Meyer (1882), Seo (1931), and Setna (1930) which shows that certain of these non-locomoter type of cilia are nervously controlled. That such cilia on some animals are nervously independent, however, is strongly indicated by the continued beating of cilia for several hours on small isolated pieces of ctenophores, mollusks, and annelids. The other function of cilia is to serve as locomotor structures. These cilia may serve as the entire locomotor system or they may act only as an aid to another type of effector system during locomotion. Since the cilia in this category must change either their rate or direction of beating as the animal changes its rate or direction of movement or must cease beating entirely, it has been assumed that they are under the control of nerves. Certain data of experimental and structural origin have been given to demonstrate the view of nervous control (Copeland, 1922; Carter, 1926). It has been suggested that these nerves are of the inhibitory type. If this be true, then it is natural to assume that the state of ciliary activity is the normal condition of ciliary protoplasm. This view is supported by Chambers (1924) who, in referring to observations made on the ciliated cells of the ovaries of sea urchins, stated (p. 258), " An active cell can be impaled on the end of a needle without interfering with the movements of the cilia. If, however, the surface of the cell at its base be pricked and ever so slightly torn, a breakdown travels rapidly over the cell. As the injury reaches the ciliated border the peripheral cilia stop beating and the further advance of this injury can be followed by the successive cessation of more and more cilia until all ciliary activity has ceased. The cytoplasm then becomes dissipated in the sea water and the cilia fall apart as motionless filaments."

That certain stains show nerves and fibers within ciliated cells in various animals is clearly stated in reports of several investigators. Meyer (1882) found nerve fibers ending in a layer of ganglion cells below the epithelium of *Polyophthalmus*. Agersborg (1923) noted neural fibers in *Melibe* extending from the pedal ganglion to the bases of ciliated, epithelial cells where these fibers joined the intracellular fibers that were connected to the ciliary basal bodies. Carter (1926) found nerve fibers passing up between the bases of the ciliated cells of the nudibranch veliger, and later (1928) he showed nerve fibrils passing from the cerebral ganglion and terminating in the ciliated cells of this veliger. He found also intracellular fibers passing from these nerve fibrils to the basal granules within the ciliated cells. Setna (1930), however, could not find definite nervous connections in the ciliated cells of *Pecten*, and Bhatia (1926), even though he found intercellular fibrils in ciliated epithelium of *Mytilus* connecting to the subepithelium, concluded that coördination of movement is effected by cell contact rather than by any specialized fibrillar mechanism. Although fibrils within the ciliated cells and fibers within the basal membrane were seen in the stained tissues of *Nephthys* (Figs. 7 and 8), there was no evidence of connection between these two sets of fibers. Even if these stained materials represent nervous elements, physiological evidence must support this view before we can be certain that the nervous system really regulates ciliary activity. That stained fibers may not be true nervous elements has been shown by Carter (1924), who concluded that the fibers of the lateral cells of *Mytilus* brought out by stains do not represent the nature of true fibers in the living cell.

Certain physiological experiments on ciliated tissue do support the view of ciliary coördination by nerves. Copeland (1922), by cutting ciliated epithelium of the foot of *Polinices*, demonstrated that impulses were carried from the subepithelial tissue to these ciliated cells. Merton (1923) produced activation of quiescent cilia on the epithelium of the snail by stimulating electrically the nerves leading to this tissue. McDonald (1927) found that the pharyngeal cilia of the frog beat more rapidly when their sympathetic nerve supply was stimulated and they beat less rapidly when their parasympathetic nerves were stimulated. Seo (1931) reported that ciliary movement in the palate of the frog and the toad were reflexly accelerated by the stimulation of the tongue or the glossopharyngeal nerves. Although these experiments do show certain facts which indicate the nervous regulation of ciliary beating, the continued rhythmic movement of cilia on isolated pieces of tissues shows automatic activity which is indicative of nervous independence. This lack of nervous regulation was demonstrated on *Nephthys* when the destruction of a ciliated cell between two other similar cells failed to interfere with the beating of their cilia. It might be said that the regulating impulses lie in the basal membrane, where definite fibers were found, and therefore the destruction of a single ciliated cell does not affect this transmitting system. Since the application of pressure, however, to the adjoining basal membrane cells failed to interrupt ciliary movement, whereas the application of the same amount of pressure to the ciliated cells did stop their activity, it is assumed that the basal membrane is not concerned in the regulation of these cilia. The results of experiments on the ciliated cells of *Nephthys* show them to be independent of any nervous control. This is in agreement with the results of Lucas (1931*a*), who stated that the ciliated epithelium of *Mytilus* is not regulated by a nervous system. Lucas (1931*b*) also cut between two active latero-frontal ciliated cells of a lamellibranch gill without producing any effect on the movement of their cilia. Furthermore, Gray (1922) came to the conclusion that the cilia of *Mytilus* are entirely independent of any nervous control.

Lucas (1932*a*) concluded from results of experiments on the latero-frontal cell of *Modiolus* that an impulse sufficient to stimulate other quiescent cilia to activity is transmissible through cells bearing quiescent cilia. This cannot be the entire activating mechanism in *Nephthys* since a cell between two active ciliated cells was destroyed and yet the cilia of these two cells continued to beat normally. Not only is the ciliary movement of *Nephthys* independent of the nervous system but the coördination of the metachronal wave is independent of this system also. Since the evidence shows the movement and the metachronal wave of cilia of *Nephthys* to be independent of the nervous system and since these cilia continue to beat in a normal manner on an isolated cell, then each cell must possess its own regulating mechanism. This view has been expressed by Worley (1933), who believes that the individual cell governs its own metachronism. Possibly a neuroid mechanism is concerned with the regulation of cilia when they are in their normal location on the animal, but this type of system cannot account for the entire coördination. The type of apparent independence of the cilia of *Nephthys* is not unusual since Gray (1924) has drawn a parallel to this in the contraction of cardiac muscle.

From the data given by others in regard to the control of various ciliated tissues and from the results of experiments on the ciliary system of *Nephthys*, it is apparent that no general statement in regard to ciliary control can be made with any degree of exactness. This controlling system is most likely different for different ciliated materials.

SUMMARY

1. The cilia of *Nephthys* are grouped into small tufts on the branchia and on the body wall of each parapodium. Each tuft is limited to a single cell and consists of 40 to 50 large compound cilia. Each compound cilium is made up of smaller units.

2. The longest cilia measured were 62 μ while the shortest ones were 5.6 μ.

3. The cilia of *Nephthys* in their effective and recovery strokes move according to the flexural type.

4. The corresponding cilia of each tuft along the branchia move synchronously while those exactly across the branchia are opposite in their phases of movement.

5. The effective stroke of the cilia is at right angles to the metachronal wave.

6. Iron hematoxylin, with or without a counter stain, showed intracellular fibrils passing from the base of each ciliated cell to the cilia. This stain showed also many fibers within the basal membrane beneath the ciliated cells. There was no connection between the fibers within the basal membrane and the intracellular fibrils of the ciliated cells.

7. Both Vom Rath's stain and methylene blue showed the cytoplasm of the ciliated cells to be granular.

8. Physiological tests indicate each ciliated cell to be independent and that it regulates its own activity.

9. Stains and the results of physiological experiments indicate the ciliary system of *Nephthys* to be independent of the nervous system. Possibly a neuroid mechanism is responsible for the ciliary coördination of these cells when they are in their natural location.

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FACTORS CONTROLLING LONGEVITY IN PROTOZOAN PROTOPLASM

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I well remember the thrill I got when I first saw a living *Amœba proteus* under the microscope; its glairy substance, evidently liquid but not mixing with the surrounding water, its enigmatic movements and method of feeding were all fascinating. Nor was the thrill lessened in any degree when I realized that this bit of living protoplasm, in all probability, had been continuously living since the beginnings of life and, barring accidents, had the possibility of an indefinitely continued existence in the future.

What is true of *Amœba* is equally true of the many thousands of species of other Protozoa, some of which are vastly more complicated. The protoplasm composing each of them and each with its specific make-up or organization has been continuously living in the past and has the possibility of living continuously in the future.

What are the factors underlying this remarkable longevity, and what are the observable processes by which this marvellous continuity is maintained? In the following pages I shall attempt to answer these questions.

Weismann, more than fifty years ago in his classical essays on "Life and Death," dealt with this problem in his characteristically suggestive way. Protozoa, he argued, are simple bits of protoplasm which in a proper medium have within themselves all that is necessary for an indefinitely continued life. They receive stimuli and food from the environment, react to the stimuli, ingest and digest the food and grow. When they reach the limit of growth typical of the species, they divide. A fully grown bit of protoplasm does not die but its living substance is parcelled out to two daughter cells each of which repeats the cycle of feeding, growth, and division. Natural death, therefore, in Protozoa, is unknown—there is no corpse.

Protozoa, he continued, are equivalent to the germ cells of Metazoa which are, likewise, potentially immortal. But germ cells upon development give rise to specialized cells, tissues, and organs which have a limited potential of vitality and these ultimately die. Natural death, said Weismann, is the penalty which Metazoa pay for the privilege of specialization.

Weismann, in common with his contemporaries, recognized what we regard as the first or primary group of factors underlying longevity, viz. a proper medium, and the fundamental metabolic activities of the organism; life without these is unthinkable. His other conclusions, with our knowledge today of Protozoa, are not acceptable. Metazoa and Protozoa are not fundamentally different but are fundamentally alike, with differences only in degree.

Fundamental and Derived Organization

The egg cell of a metazoön is a small, apparently homogeneous sphere of protoplasm which will develop into exactly one type of organism. It has a specific make-up or fundamental organization which differs from the fundamental organization of every other type of egg cell. Upon stimulation it develops by continued cell division into organs and structures of a specific type of adult with a characteristic derived organization which is the basis of classification. The derived structures constitute the soma and together they protect and nourish the germ plasm with its fundamental organization within. Upon exhaustion of the potential of vitality of the derived organization the individual dies a natural death and its germ plasm, without protection and nourishment, is killed. The derived organization in the vast majority of Metazoa is not turned back or resolved again into the germ plasm with its fundamental organization.

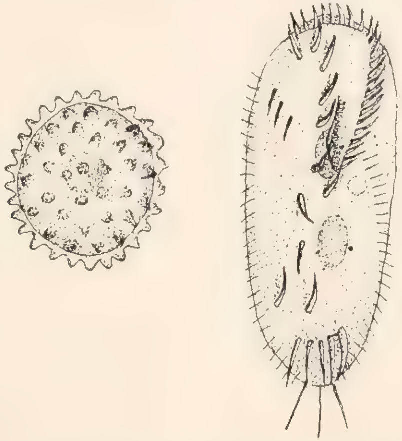

Fig. 1. *Stylonychia pustulata.* Encysted organism with its fundamental organization, and the adult organism with its derived organization.

Let us now look at a protozoön, preferably a ciliate with its fairly complex organization. Like most ciliates, it may be found in an active vegetative, or in an encysted, quiescent condition. The cyst of any species of the genus *Stylonychia* is a small spherical structure with a characteristic corrugated external surface (Fig. 1). Within it is a ball of apparently homogeneous and structureless protoplasm. Like an egg cell, it has a specific fundamental organization which will develop into one specific type of adult and into nothing else. Place such a cyst in a suitable nutrient medium; soon the cyst wall softens and becomes permeable to water and oxygen and the fundamental organization develops quickly into an active organism with its characteristic adult derived organization (Fig. 1). Unlike a metazoön, the derived structures are not formed by cell division but are differentiated parts of the living fundamental organization itself, just as an egg cell might bud out derived structures continuous with its own protoplasmic make-up. Like the soma of a metazoön, these derived structures are vitally important to the organism in its metabolic activities. In *Stylonychia* they consist of macronuclei, mouth gullet and gastric vacuoles, peristome, anal opening, undulating membranes, membranelles and various types of cirri, contractile vacuole and canals. They are formed from and are continuous with the fundamental organization.

In different types of Protozoa derived organizations have a widely varied significance both as to origin and as to function. With Doflein we may group them in two categories—the euplasmatic and the alloplasmatic. The former are differentiations of the living protoplasm such as we find in pseudopodia of the Sarcodina, epimerites of the Gregarinida, or in the various motile and cortical organs of the Infusoria. The latter are usually products of secretion or of precipitation by the living protoplasm and are lifeless matter as in shells, tests and skeletons of Foraminifera, Radiolaria, etc., or as in gelatinous secretions (pseudochitin or tectin of Bresslau) forming houses, cups, stalks, etc. of flagellates and Infusoria. Such alloplasmatic structures of Protozoa are equivalent to the skeletal elements of the Metazoa soma and, like them, when once formed, are irreversible. With them we have justification for challenging Weismann's denial of a corpse in Protozoa. Forming an important part of the soma, a shell of a foraminiferan or a skeleton of a radiolarian is left behind with a small bit of residual protoplasm when the fundamental organization breaks up into a multitude of germ cells which leave the old cell behind as a relic of the old generation. The sea bottom is covered fathoms deep with such corpses of previously living individuals.

The euplasmatic structures of the derived organization, on the other hand, have no such fate. Formed by and from the fundamental organization, they have a limited potential of functional activity, then are withdrawn or absorbed into the fundamental organization and new structures are formed to replace them. This phenomenon of absorption and replacement termed *reorganization* occurs at critical periods in the life history and, with its different manifestations, constitutes the second and in some ways the most important group of factors underlying longevity.

The fundamental organization of a ciliate is undoubtedly found in its purest form in the permanent cyst. Prior to encystment the motile organs and other cortical differentiations are absorbed; the macronucleus fragments and the fragments are absorbed; water and waste matters in the endoplasm are discarded and an impervious covering membrane, the cyst wall, is secreted from the peripheral zone of protoplasm. The micronucleus divides and one of the products will form a new macronucleus. The fundamental organization, reduced to its lowest terms, devoid of water and protected by its cyst, may be dried and kept in the dried state for months or even years and without impairment of its potential vitality. The specific organization, presumably made up of nucleoproteins, proteins, carbohydrates, fats, and salts, in a definite combination, remains intact.

Ultimately, in a favorable environment, water and oxygen are introduced through the now permeable cyst wall and metabolic activities begin. Through these, structures are developed which were not present before. These are formed mainly in the cortex but the new macronucleus develops rapidly in the endoplasm. The young organism, after whirling around in its cyst, finally emerges and begins a new life cycle with high vitality. With its full complement of cell organs the physiological activities of the young organism begin at once. Reactions to stimuli, movement, ingestion of food, secretion of digestive fluids, digestion and assimilation of food, defecation of undigested remains, excretion of metabolic waste, and a multitude of other internal reactions, all go on in full swing. The organism grows.

The varied activities do not leave the protoplasm as it was when it left the cyst. Chemical processes of metabolism result in substances not present before and, transient or stable, they play their part in further activities. Physically, changes in phase in the polyphasic system are probable although not demonstrated. In short, the fundamental organization is constantly changing and is never twice the same in an active organism. Furthermore, the changes are cumulative and finally a condition is set up when some drastic reaction is essential for continued life. Reorganization, or a return of the protoplasm to the fundamental organization, now occurs and is consummated by the phenomena of cell division.

Reorganization by Division

The changing make-up of the protoplasmic organization as a result of continued metabolism from the end of one division to the advent of the next is difficult to demonstrate on any morphological basis. Physiologically, however, it is possible to show that the organization is not equally responsive at all stages between divisions and the implication is that changes do take place. A simple merotomy experiment illustrates this (Calkins, 1911). A marine ciliate, *Uronychia transfuga,* is cut transversely through the center so that half of the macronucleus and the micronucleus are in one fragment, the other half of the macronucleus and no micronucleus in the other fragment. If the operation is made on an individual three to five hours after its last division, the fragment with a micronucleus regenerates perfectly but the fragment without a micronucleus, while it may live for four or five days, never regenerates the missing structures. The same result is obtained if the organism to be cut is from ten to fifteen hours old, but if the individuals cut are older than this we get an increasing percentage of perfect regenerations of the amicronucleate fragments. If the individual is cut at the age of twenty to twenty-two hours, the complete regeneration of this fragment, although without a micronucleus, is invariable. This indicates a progressive differentiation in respect at least to the power of regeneration and to that extent a change in organization. Furthermore, if one individual is similarly cut while the two daughter cells at division are still connected, or shortly afterwards, the amicronucleate fragment will not regenerate the missing parts. This indicates that the condition which underlies the power to regenerate is lost with the processes of division and is not regained until the young cell has undergone a considerable period of normal metabolism.

It is evident that the period of full inter-divisional maturity is one of high catalytic activity shown by all parts of the organism and manifested by division of cellular elements and by other significant processes. In regeneration it is shown by the division of the basal bodies which underlie the formation of the regenerated cirri. In normal division it is manifested by remarkable reorganization processes in the macronucleus and by absorption of structures of the euplasmatic derived organization which are replaced by new ones.

Division of the cell thus is by no means a simple process but a vital phenomenon of the utmost importance and is accompanied by far-reaching changes in organization. These changes begin with the macronucleus, which is probably the most important of all the derived structures. The macronucleus arises from a product of an early division of the micronucleus at encystment or endomixis, or from the amphinucleus after conjugation. In an ex-conjugant it is not stained at first with the usual nuclear dyes but nucleic acid (chromatin) is manufactured in it and when it is ready for the first division it is filled with intensely staining, uniform granules of chromatin exactly like the macronucleus after encystment. With the ensuing metabolic activities the homogeneous structure changes. In *Uroleptus*, where the history has been carefully followed, a different type of granule soon appears—the so-called X-granules. These do not stain with basic dyes and are hydrolyzed out with the Feulgen reaction. On the approach of division these become more numerous in each of the eight or more macronuclei. They apparently act as catalysts, for a characteristic nuclear cleft (Kernspalt) appears in each nucleus, associated with one large granule or with a group of two or more. Separated by the cleft, the larger portion of the nucleus now contains large-sized, uniform, and deeply-staining chromatin granules while the smaller portion, distal to the cleft, contains smaller and less deeply-staining granules (Fig. 2). The latter portion is cast off in the cytoplasm together with the nuclear cleft and any remaining X-granules. These cast-off parts disappear, evidently by absorption in the cytoplasm, while the remaining portions now unite to form at first an irregular fused mass which condenses to form the single ellipsoidal macronucleus, with deeply-staining chromatin granules, ready for division.

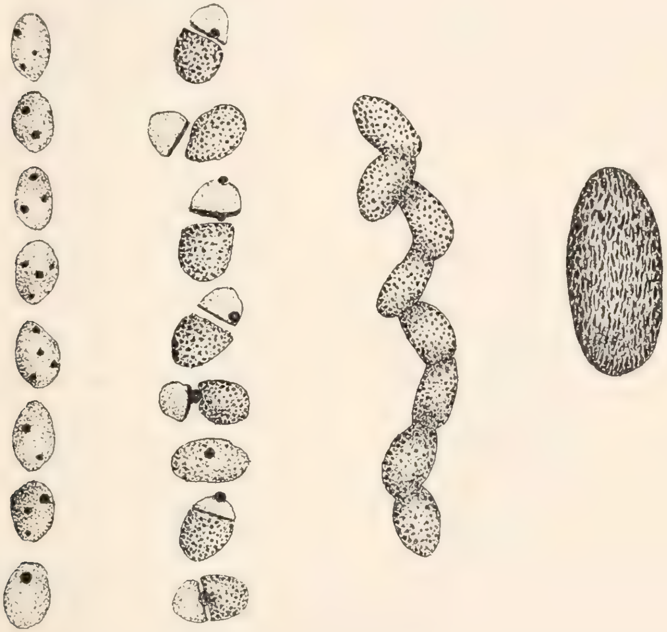

Fig. 2. Macronuclei of *Uroleptus halseyi*. X-granules in the eight macronuclei which act as catalysts whereby approximately one-third of each macronucleus with modified chromatin is cast off into the cytoplasm. The other parts, with re-organized chromatin, unite to form the single division nucleus (see Calkins, 1930).

Analogous processes of macronuclear chromatin elimination, which I have elsewhere referred to as evidence of "nuclear purification," occur in different ways in other ciliates. Kidder (1933) described a core of modified chromatin in the center of the macronucleus of *Conchophthirius* (*Kidderia*) *mytili*. This core condenses into a small deeply-staining ball which, upon division of the macronucleus, remains for a time in the connecting strand between the daughter nuclei but ultimately disappears by absorption in the cytoplasm (Fig. 3). A similar extrusion, referred to only incidentally by Rossolimo and Jakimowitsch (1929), occurs in *Conchophthirius steenstrupii*. Here it is in the form of a finely granular substance which comes from the macronucleus and remains for a time between the nuclear halves after division but ultimately disappears in the cytoplasm.

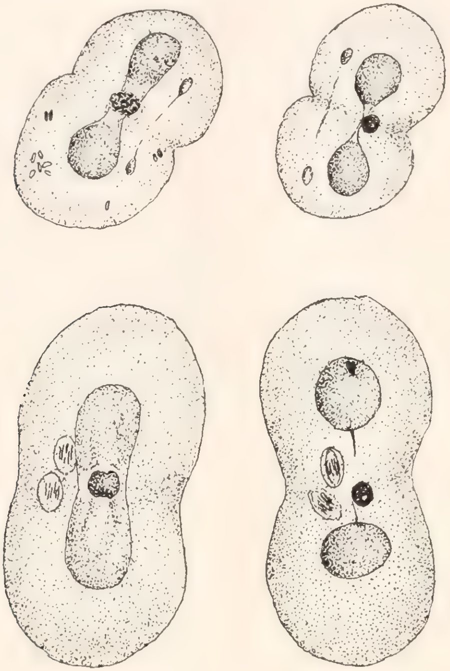

FIG. 3. *Conchophthirius anodontæ* (upper row) to show segregation and emission of the extrusion chromatin. *Kidderia* (Raabe) *mytili* (lower row) in same stages. From preparations presented by Dr. G. W. Kidder (see Kidder, 1933).

There is reason to believe that this phenomenon has something to do with re-establishing full metabolic power of the macronuclear chromatin, possibly by the elimination of waste products of chromatin activity.

Another process of macronuclear reorganization which does not involve the actual elimination of nuclear substance occurs in the families Aspidiscidæ and Euplotidæ of the hypotrichous ciliates. At the approach of division in *Aspidisca*, according to Summers (1935), a granule appears at about the center of the convex side of the C-shaped macronucleus. The substance of the granule stretches across the macronucleus in two bands which form a V with the remains of the granule at the apex. The two arms of the V now move in opposite directions, traversing the entire macronucleus and disappearing at the two ends (Fig. 4). The increasing zone between the two arms is filled with deeply-staining chromatin granules which are quite different in appearance and in staining capacity from the chromatin in parts of the nucleus which have not been traversed by the bands. After disappearance of the bands at the ends, the nucleus condenses to form the typical division macronucleus of *Aspidisca*. Here again, therefore, there has been a change in the physical condition of the chromatin and a change that

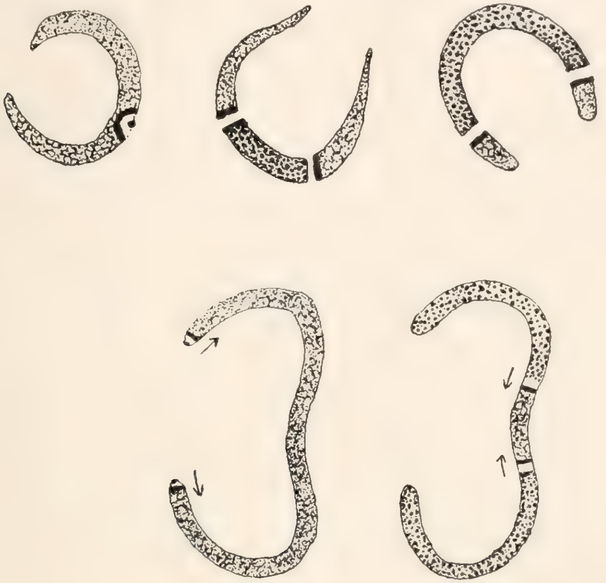

FIG. 4. *Aspidisca lynceus.* (Top row.) Macronucleus with origin of the reorganization bands in center, and two phases in their migration towards the ends. From preparations presented by Mr. F. M. Summers (see Summers, 1935).

Euplotes patella (lower row); the reorganization bands originate at the two ends of the macronucleus and pass through the nucleus until they meet in the center. Chromatin between the bands is in the form of a reticulum. This is changed into discrete granules with passage of the bands (see Turner, 1930).

is brought about through the activity of nuclear cleft substances acting as catalysts.

A similar macronuclear reorganization is brought about in *Euplotes.* Here, as a division period approaches, a nuclear cleft develops at each end of the long C-form macronucleus (Fig. 4). These two clefts now move towards each other, traversing the entire nucleus until they meet in the center where they disappear. The middle portion between the two clefts contains chromatin in the form of a reticulum, but the ends, after the clefts have passed, contain large, independent and deeply-staining chromatin granules indicating that the chromatin undergoes some kind of physical change with the passing of the bands. After the bands disappear, the nucleus shortens into a rod-shaped structure now ready for division.

This remarkable phenomenon has been described by numerous observers. Although previously observed, the clefts, or "reorganization bands" so-called by Yocom (1918), were first adequately described by Griffin (1910) in *Euplotes worcesteri.* He called them "reconstruction bands" and described them as made up of two parts, one of which, a proximal part turned towards the center of the nucleus, is deeply and uniformly stained. Griffin regarded this as a "solution plane" in which the chromatin is completely dissolved. The second portion, which he called the "reconstruction plane" is in close contact with the solution plane and in most cases appears uniformly clear and unstained and with no trace of structure. In some cases he found what appeared to be linin fibrils on the distal ends of which minute chromatin granules were seen; these enlarge rapidly into intensely staining discrete granules characteristic of the distal ends of the nucleus. Griffin believed that the old chromatin is completely dissolved in the solution plane and is reformed without the impurities of the old chromatin—a process of nuclear rejuvenescence.

Yocom (1918) gives a somewhat different interpretation of these enigmatic structures which he calls "reorganization bands" in the related species *Euplotes patella.* The two parts of each band are present as in the description above, but Yocom holds that the chromatin does not go into solution in what Griffin calls a solution plane, but "appears there as closely packed granules from which they pass over the reconstruction plane not as precipitated chromatin but as granules which have undergone some sort of physical and possibly chemical reorganization."

Turner (1930), also working on *Euplotes patella,* agrees with Griffin regarding the absence of granules in the solution plane and considers the substance in it to be in the physical state of a colloidal solution. The chromatin reticulum in the center of the nucleus he interprets as in the continuous phase while the nuclear karyolymph is dispersed. After action in the reorganization bands this condition is reversed, the chromatin granules being in the dispersed phase and the karyolymph in the continuous.

These phenomena indicate that, at division, a peculiar type of catalysis occurs in the macronucleus whereby the chromatin undergoes some kind of reorganization which leaves it in the condition of a young organism ready to divide. They are, moreover, expressions of a general wave of reorganization which passes over the entire organism, resulting in a more or less complete new set of derived structures for both anterior and posterior daughter cells. The underlying cause of this periodic and cataclysmic phenomenon is unknown but the facts are unmistakable.

The first careful observations on the happenings to the cortical structures of the derived organization were made by Wallengren (1900, 1901, 1902). Prior to this, Stein (1859), Sterki (1878), and others had observed and maintained that all of the motile organs and oral parts are renewed at division in a number of ciliates (*Holosticha, Stylonychia, Gastrostyla, Uronychia,* etc.). Wallengren carefully described the process for each of the cirri of *Stylonychia* and other ciliates and, with minor variations, fully confirmed the earlier observations. The latest observer, Turner (1930), finds that not only are all cirri of *Euplotes patella* absorbed and renewed, but that the coördinating fibers from the anal cirri are also included in the loss and regeneration of the elements of the neuromotor system. Contrary to previous observers, he finds that in *Euplotes* the original cell mouth is not renewed during division but continues to function throughout the process. Whether or not it is renewed shortly after division is not yet satisfactorily determined.

The phenomena described above as occurring in the relatively few types of ciliates that are cited probably occur in the same or in modified form in all types of Infusoria. Metabolic activities during interdivisional periods result in a changed organization in which a general wave of catalysis sweeps over the organism and ends in division of the cell as a whole. One effect of this wave is a restoration of the old macronucleus to its original post-cystic condition; another effect is the withdrawal of the structures of the old euplasmatic organization back into the fundamental organization and their replacement by new derived structures. Here, as with de-differentiation described by Child, the reorganization processes result in an increased rate of metabolism.

Flagellates, Sarcodina, and Sporozoa are less spectacular than the ciliates in phenomena of reorganization by division. Nevertheless there are some striking cases of reduction, absorption, and re-formation of derived structures. In flagellates division of the cell is longitudinal sc that differential regeneration, characteristic of ciliates, is absent.

While there are many cases on record where the old flagellum is retained by one of the daughter cells at division and a new flagellum grows out from the divided blepharoplast in the other daughter cell, there are many cases on record of absorption of the old flagellum or flagella, and re-formation of flagella from centrioles or blepharoplasts. The latter usually arise by division of the endobasal body of the nucleus as in *Spongomonas*, *Trichomonas*, etc.

One of the best-supported cases in the latter category is the genus *Lophomonas*, an intestinal parasite of the cockroach. Here the spherical (*L. blattarum*) or elongate (*L. striata*) cell has a prominent brush of flagella at the anterior end. Each flagellum comes from a basal body and each basal body from a short rod-form blepharoplast. The group of blepharoplasts form a collar at the anterior end of the cell. Each blepharoplast gives rise posteriorly to a fibril (rhizoplast) and the group of fibrils form a basket or calyx in which the nucleus lies. Posterior to the nucleus the fibrils come together to form an axial strand which runs to the posterior end of the cell.

When ready for division, the nucleus emerges from its basket, centrioles derived by division of the endobasal body appear on the outside of the nuclear membrane, and a spindle is formed between them. The nuclear complex now migrates to the posterior end of the cell and a minute group of blepharoplasts arises from each centriole. The nucleus divides and the two daughter nuclei, each with its newly-formed group of blepharoplasts, migrate towards the anterior end of the old organism, one on each side of the division plane. The old aggregate of flagella, basal bodies, and blepharoplasts disappears by absorption in the protoplasm of one of the daughter cells, each of which has a newly-formed group of flagella, collar, calyx, and axial strand. In principle this is the same type of phenomenon as in ciliates and recalls the loss of the old mouth and oral basket of trichites, and the formation of two new mouths and baskets during the division of *Chilodon uncinatus* as described by MacDougall (1925).

In the majority of the Sarcodina and Sporozoa binary division is not the usual mode of reproduction. Where it does occur, as in Amœbidæ, noticeable derived structures are absent and reorganization, if it occurs, is confined to the cytoplasmic make-up of which we have little evidence. In Testacea there is a modified type of division, so-called budding division, during which water is absorbed by the protoplasm of the old cell. This is accompanied by lively cyclosis and protrusion of a protoplasmic bud from the shell mouth. This bud grows, assumes the shape of the parent shell, and secretes its own chitinous membrane on which foreign particles (Arcellidæ) or plates manufactured by the parent protoplasm (Euglyphidæ) are glued. Apart from the withdrawal of old pseudopodia there is no visible evidence of reorganization. In Heliozoa at division, pseudopodia with their axial filaments are drawn in and new ones are formed by the daughter cells.

In the vast majority of Sarcodina (Radiolaria, Foraminifera, and Mycetozoa) and in most Sporozoa, binary division is replaced by multiple division. Nuclei divide repeatedly and a portion of the cytoplasm is ultimately parcelled out to each of the daughter nuclei. The minute cells thus formed leave the parent organism as swarm spores. Metabolic products, waste matters, and structures of the derived organization are abandoned and the shells and skeletons alone record the existence of the previously living cells. Each spore has only the fundamental organization together with a new potential of vitality; it is equivalent to the reorganized daughter cell of a divided ciliate.

Inadequacy of Reorganization by Division

It might seem that the method of restoring protoplasm to its fundamental organization by division would suffice to explain the indefinite longevity of Protozoa. This, however, is not the case. It is true that in animal flagellates this is the only method known, but it is not true of other groups. In ciliates the power to divide, if other methods of reorganization are prevented, gradually weakens; the inter-divisional periods are progressively lengthened; degeneration of the derived structures finally results. The micronucleus hypertrophies (Calkins, 1904) or disappears (Maupas, 1888) and the motile organs are lost (Maupas, 1888). In *Uroleptus mobilis* the chromatin of the macronucleus ultimately disappears and only a few large X granules remain. The protoplasm dies from what appears to be old age.

The effects of continued division in ciliates, with the exclusion of other methods of reorganization, are clearly shown by the method of isolation cultures. A single individual, preferably an ex-conjugant, is isolated in a suitable medium in a culture dish. The next day such an individual, for example a *Uroleptus mobilis*, is represented by four or eight daughter individuals, the number depending upon two or three divisions of the original one isolated. Five of these individuals are separately isolated in culture dishes, thus starting five lines all of which represent the protoplasm of the original ex-conjugant. A single individual is isolated daily from each line and the daily record of divisions is kept for all lines. The total number of divisions in all lines is averaged for 10-day periods. The division rate is taken as a measure of vitality and the history of vitality is obtained by plotting the consecutive 10-day averages on a graph where the ordinates represent the number of divisions, the abscissas the consecutive 10-day periods. Such a graph for *Uroleptus mobilis* is shown in Fig. 5. There is a high initial vitality which gradually wanes through some 360 ± divisions during 300 ± days. In these isolation experiments the individuals do not encyst nor do they conjugate, hence division is the only means of reorganization. That division is not effective in checking the waning vitality is shown by the fact that, without exception, all such cultures die.

There is no doubt that the macronucleus is the most important of all derived structures of the ciliate organization. With each division of the cell, as shown above, it undergoes a process of reorganization whereby the chromatin is restored to a virile condition. With *Uroleptus mobilis*, in isolation cultures, this is repeated upwards of 350 times until reorganization, if it occurs, is ineffective and the protoplasm dies. It is probable that the macronucleus, like all derived euplasmatic structures, has a limited potential of activity. Other derived structures are periodically renewed from the fundamental organization. So too, under favorable conditions, is the macronucleus, the renewal taking place during the processes of endomixis and of conjugation.

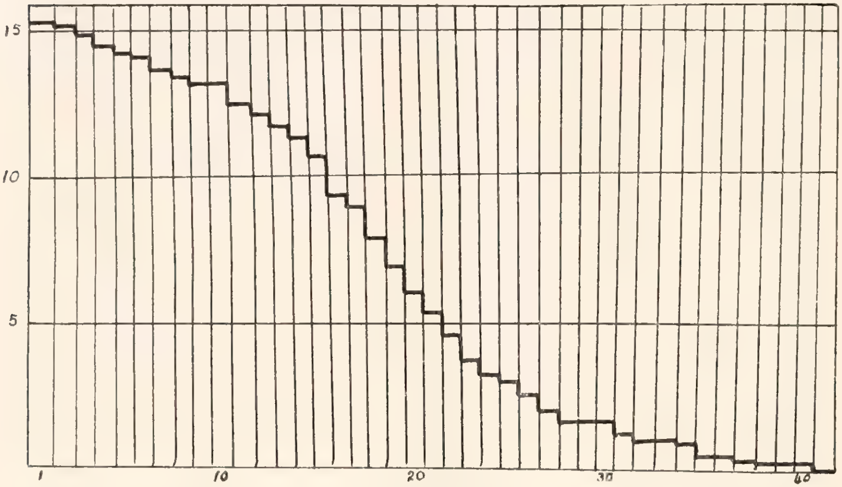

FIG. 5. Vitality graph of *Uroleptus mobilis*. A composite graph of twenty-three series averaged for successive 10-day periods.

In isolation cultures, if the extra individuals which remain over after the daily isolation is made on any day are placed in a larger container with abundant food, a so-called "encystment test" or "conjugation test" is started. Here the individuals multiply by division until there may be thousands in the container. In the early stages of the life cycle of *Uroleptus mobilis* all such individuals ultimately die of starvation, but after approximately a month or six weeks after conjugation such tests result in an increasing number of encystments. A type of catalysis which is not seen in the division phenomena is now manifested. The macronucleus is broken down by fragmentation and the fragments are absorbed in the protoplasm while a new macronucleus is formed from a product of micronuclear division. Other structures of the derived organization are absorbed, waste matters and water are voided to the outside, and a cyst membrane is formed within which the organism may remain in a desiccated condition for months. When it is brought out of the cyst and established in isolation culture, it has an optimum vitality and passes through a complete life cycle exactly like that of an ex-conjugant. This phenomenon of endomixis, except for encystment, is the same as endomixis in *Paramecium aurelia* originally described by Woodruff and Erdmann (1914). Endomixis thus brings about a more far-reaching and a more complete reorganization than does division, and the important derived structure, the macronucleus, is renewed.

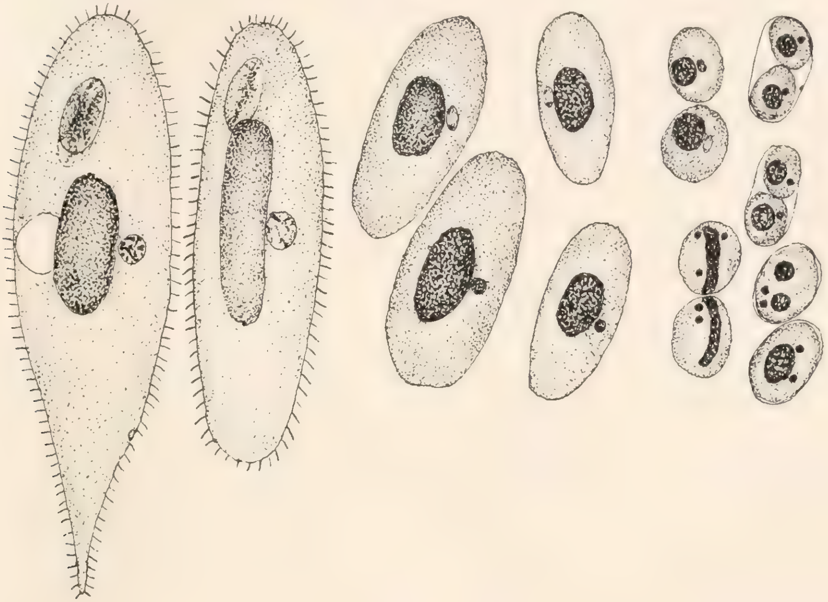

FIG. 6. *Glaucoma* (*Dallasia*) *frontata.* History of a normal vegetative individual with successive divisions resulting in copulating gametes and encysted zygotes (see Calkins and Bowling, 1929).

An interesting and illuminating variation, which I regard as equivalent to endomixis, is shown by the ciliate *Glaucoma* (*Dallasia*) *frontata*. At one stage in its life history, a stage corresponding to the period of encystment in *Uroleptus, Glaucoma frontata* loses its normal form and its normal movement and becomes elongate and cylindrical, darting about in the medium with unusual speed (Fig. 6). Soon its mouth and complicated oral membranes are absorbed and it divides into two ellipsoidal individuals. These divide into four, the four into eight, and the eight into sixteen individuals without any intervening growth. These sixteen have no resemblance to the earlier parent form but each has a macronucleus and a micronucleus which has undergone reduction divisions and is haploid as to chromosomes. The sixteen are associated in pairs which become surrounded by a cyst membrane within which they fuse (Calkins and Bowling, 1929). This series of divisions is comparable with the process of endomixis in *Paramecium* with the exception that in the latter the micronuclei divide without division of the cell body. It further indicates that in ciliates generally, endomixis is a reminiscence of a gamete brood.

Reorganization by Conjugation

The longevity of a ciliate's protoplasm in the past is evidenced by the fact that it is before us today and has the possibility of indefinite longevity in the future. We know very little about the ultimate secrets of protoplasmic organization which underlie continued life, but we can analyze and control some of the conditions under which it keeps going. A proper environment and adequate food are essential factors. These lead to growth and to reproduction by division. We have seen above that the processes of division involve reorganization and the return of the protoplasm to a labile condition with a higher potential of metabolic activity. The periodic recurrence of these processes might well be adequate to ensure the longevity of the protoplasm, as seems to be the case with animal flagellates. It may well be the case, also, with some ciliates, but it must be remembered that the central organoid of metabolic activity—the macronucleus—is itself a derived structure and, presumably like other derived structures, is subject to absorption and replacement by a new macronucleus derived from the fundamental organization. This replacement does not occur at division, but, as shown above, it undergoes its own reorganization which we have likened to a nuclear purification. At endomixis, however, it follows the fate of other parts of the derived organization; a new one is formed from the fundamental organization and the result, again, is an increased vitality of the protoplasm. This phenomenon, recurring every thirty days, ensured the longevity of *Paramecium aurelia* in Woodruff's hands for many years and through some thousands of generations by division. This phenomenon, indeed, as Woodruff states, may be adequate to ensure indefinitely continued life in the future, as division alone is adequate for flagellates.

In a conjugation test made with *Uroleptus* when the series is sixty or more days old the individuals multiply by division until there may be thousands in the container. After four or five days the bacterial food is diminished or even exhausted, and, if the race is "mature" an epidemic of conjugation occurs. Two individuals fuse at the anterior ends and remain united for about twenty-four hours (Fig. 7). They then separate and each of the two begins a process of reorganization which lasts for four or five days. If such an ex-conjugant is isolated, it will give rise to a new series and its progeny will pass through all the stages of vitality that the parent series passed through. At the outset its vitality will be the same as the original vitality of the parent series, but it will be greater than that of the parent series at the time of conjugation. It passes through the same history of waning vitality and its protoplasm finally dies, although this may be months after death of the parent protoplasm. This latter fact led Woodruff and Spencer (1924) to conclude that the effect of conjugation is to impart a certain "sur-

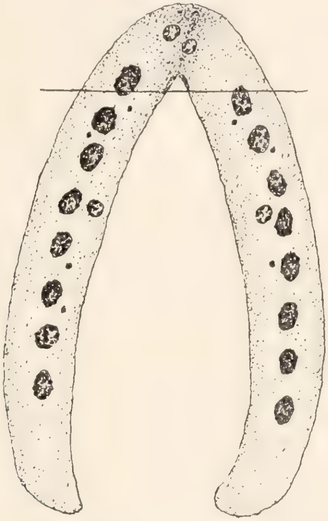

FIG. 7. *Uroleptus mobilis,* conjugating. The wandering pronuclei are passing each other at the anterior end. The line indicates the plane of cutting in merotomy experiments (see Calkins, 1921).

vival value" to each of the ex-conjugants. Vitality, however, means more than survival or life continued beyond that of the parent protoplasm. There are at least two factors involved—intensity of vitality and endurance (Calkins, 1920). A series living 100 days and dividing 150 times has an intensity of vitality represented by an average of 1.5 divisions per day. Another series living 300 days and dividing 450 times has the same average intensity of 1.5 divisions per day, but no one would say that the two series have the same vitality. Endurance (survival value) and intensity must be taken together in order to measure vitality, and both are increased as a result of conjugation.

These experiments with *Uroleptus,* continued for more than ten years and with 146 different series, invariably gave the same result. Each series lived for a period of from three months to a year or more and all were descendants of a single bit of protoplasm making up the body of one *Uroleptus* cell.

There is little reason to doubt that some change in the protoplasmic make-up occurs with continued metabolism through many successive generations by division. In isolation cultures it is manifested by an increasing time interval between divisions, i.e., a decreasing division rate, and by a change in the physical properties of the protoplasm whereby fusion of cells, total or partial, is possible. A type of catalysis not manifested earlier in the life history is set up. The cortex is liquified, usually in the region of the peristome, and two individuals fuse in conjugation at this region. In some cases the entire cortex is similarly liquified and individuals will fuse at any point on the periphery. I have seen no less than nine *Paramecium caudatum* thus fused into one amorphous mass.

The stimulus of normal fusion results in activities not manifested before. The micronuclei divide as they do in endomixis and their divisions involve reduction in number of chromosomes and a mutual interchange of pronuclei which meet and fuse. This activity of the micronuclei is generally interpreted as a reminiscence of an ancestral gamete brood which is realized in *Glaucoma frontata* and in gregarines. In the latter each individual of a syzygy forms a brood of gametes which copulate with similar gametes from the other individual of the pair.

Coming at the end of a vegetative period during which single or multiple divisions may have occurred, such gamete broods indicate a change in organization which, if copulation does not occur, results in death of the gametes. In other words, gametes are so specialized that they require a complemental combination to ensure normal metabolism. In some, differentiation has been in the direction of greater constructive activity resulting in a food-stored macrogamete or egg cell; in others, in the direction of more catabolic activity resulting in a brood of microgametes or sperm cells as in Coccidiomorpha.

The happenings at conjugation in ciliates need only be recapitulated, for the phenomena are so well known that detailed description is unnecessary. Shortly after fusion of the anterior ends the micronuclei in both individuals begin a series of maturation divisions. The third division gives rise to the gametic nuclei. One of a pair of these remains in situ, the other migrates through the protoplasmic bridge, unites and forms an amphinucleus with the quiescent gametic nucleus of the other organism. A mutual fertilization results, the two wandering pronuclei in *Uroleptus* passing each other at one stage at the apex of the united pair. After union of the wandering and the quiescent pronuclei the two individuals separate, the amphinucleus divides twice, and one of the products forms a new macronucleus which, after four or five days, is ready to divide. Another product forms a new micronucleus. In the meantime the old macronucleus begins to degenerate and it ultimately fades away in the cytoplasm. The other structures of the derived organization of the old organism are absorbed and replaced by new ones. With this new make-up the young organism starts a new life cycle with an optimum of vitality.

Through these phenomena of conjugation an old protoplasm with low vitality is made over out of its own contained substances into a new protoplasm with high vitality. What explanation of this remarkable change in vitality can be given? The only apparent differences between the happenings at division and those at conjugation are: (1) fertilization, and (2) loss and replacement of the old macronucleus. Renewed vitality after conjugation may be due to one or the other or to both of these phenomena. Conjugation differs from endomixis only in catalysis of the cortex and in amphimixis. Fertilization, with its sequelæ of hereditary possibilities, is assumed to be the raison d'être for all sorts of subsequent peculiarities, but for the phenomenon of increased vitality under consideration a very simple experiment with *Uroleptus mobilis* shows that fertilization, with amphimixis, has nothing to do with the problem. A conjugating pair is cut across the apex with a scalpel. One individual of the two thus separated is fixed and stained to show the stage in conjugation when cut; the other individual is cultivated in an isolation culture dish. The experiment is particularly significant when the two wandering nuclei are cut off with the apical piece (Fig. 7). The cultivated individual goes through exactly the same processes as though conjugation had been completed in a normal manner; the old derived structures, including the macronucleus, are absorbed and replaced; the same characteristic renewal of vitality, and the same normal life cycle occur, but amphimixis had been prevented—conjugation had been turned into endomixis (Calkins, 1921).

The second alternative, viz., the loss and replacement of the old macronucleus together with that of the old derived structures generally, remains as a possible explanation. This phenomenon is common to endomixis, conjugation, and conjugation merotomy, and in all cases a renewed vitality results. It does not happen in division although, as shown above, characteristic changes occur in the macronucleus. With its disintegration and absorption in the cytoplasm a new supply of nucleo-proteins is distributed in the cell and this may be a potent factor in the new vitality. Added to this is the fact that an entirely new and powerful organ of the cell—the macronucleus—has been supplied from the fundamental organization. The majority of the derived structures of the organism have a relatively short life, being absorbed and renewed at division (cirri and other motile organs); others, such as membranelles and oral structures, apparently have a longer life (especially anterior cells at division; posterior daughter cells always have newly-formed oral structures where the mouth is anterior). Of all derived structures the macronucleus has the longest life; it divides at cell division and may remain functional through entire life cycles including many hundreds of generations by division. It is the chief metabolic agent of the cell, while, in amicronucleate races, it is the only nucleus present. Yet it apparently lacks the possibility of continued life which the micronucleus possesses. It is essentially somatic in character and, like the soma of Metazoa, it ultimately wears out; if not replaced, the rest of the cell, including the micronucleus, dependent upon it, dies with it. It is quite possible, indeed I regard it as probable, that the gradual deterioration of this important organ of the ciliate cell is the underlying cause of waning vitality and ultimate death of protoplasm in isolation cultures.

Summary and Conclusions

The factors controlling longevity in protozoan protoplasm may be summarized and enumerated as follows:

1. A suitable environment with an adequate food supply.
2. The metabolic activities of the individual producing a constantly changing organization.
3. The catalytic processes of cell division whereby a changed organization is restored, in part, to the fundamental organization from which new derived structures are formed.
4. The catalytic processes of endomixis whereby a changed organization is more completely restored to the fundamental organization from which new derived structures, including the macronucleus, are formed.

5. The catalytic processes of conjugation, affecting the entire organization but beginning with the cortex, whereby a changed organization is fully restored to the fundamental organization from which a complete new set of derived structures, including the macronucleus, is formed. Here, also, is the opportunity for a change, through amphimixis, in the fundamental organization, and with it the possibility of variations.

6. Encystment, associated in most cases with endomixis, whereby the fundamental organization, in a dried state, may withstand unfavorable environmental conditions for long periods.

The line of thought developed above brings us back rather closely to Weismann's interpretation of Protozoa as having within themselves all that is necessary for an indefinitely continued existence. He was undoubtedly right, but when we consider the complexity of the processes involved in this continuity we are forced to the conclusion that the generalization should not be limited to the Protozoa but is equally true, with different degrees of complexity, in Metazoa and, indeed, in all other living things. His further assertion that Protozoa are equivalent to the germ plasm in Metazoa is true only in part. Had he limited it to the fundamental organization as we find it in the encysted organism, or periodically during the life history, we would find no objection to the generalization. Such an organism, like the egg cell, undergoes development, and new structures, often highly specialized and complex, are formed. Such structures constitute the soma which is analogous to the soma of Metazoa. Unlike the latter, however, the protozoan soma is reversible in greater or less degree and may be periodically renewed. When the conditions necessary for such reversion are inaccessible, as in isolation cultures with ciliates, the protoplasm dies a natural death, as in the case of the Metazoa, through waning of the potential vitality of the derived organization.

We cannot agree with Maupas' conclusion expressed in the title of his great work, "*Sur le rejeunissement karyogamique chez les ciliées*" (1889), for the old macronucleus is not rejuvenated in most cases at least in conjugation but is replaced by an entirely new one. It focusses attention, however, upon the micronucleus as the last stronghold of the fundamental organization in the struggle for continued existence.

Woodruff was right in the conclusion in his and Erdmann's paper of 1914, "A Normal Periodic Reorganization Process without Cell Fusion in Paramecium." It is probable that endomixis, with cell division, is adequate for continued existence of some ciliates, as division alone is adequate for continued life of animal flagellates. It is not, however, a specific phenomenon of Protozoa, for its counterpart, parthenogenesis, is characteristic of many Metazoa.

Fertilization in Protozoa is an expression of an almost universal phenomenon of life. In all cases it involves the fusion of two minute bits of the protoplasmic fundamental organization. These may be in the form of isogametes or anisogametes, according to the type of specialization, or in the form of minute parts only of the fundamental organization, as in pronuclei of ciliates in conjugation, or pronuclei in Cnidosporidia. In Metazoa, and in Coccidiomorpha amongst the Protozoa, fertilization involves a stimulation to development which is inhibited through specialization of the gametes. Its significance in protoplasmic longevity is problematical. For the majority of Metazoa and for Coccidia, it is, indeed, obligatory, but for many Protozoa, e.g., animal flagellates and after merotomy of conjugants in ciliates, it is quite unnecessary for continued vitality. In general its effect seems to be the restoration of the fundamental organization to a balanced condition through the union of diversely specialized gametes, together with the formation of new combinations which may result in different types of derived organizations.

Little more can be said about the fundamental organization. In the thousands of species of Protozoa each one has its own specific type of organization—and no one knows what this specificity is. Through the ages each such specificity has been retained and maintained. Each bit of the fundamental organization goes through the same series of activities—response to stimuli, food ingestion and digestion, growth. Hundreds of activities go on unseen in its substance and the organization changes with every one such activity; structures of the derived organization are formed as a result of metabolic activities and all of them, except the alloplasmatic, are periodically reversed and restored to the fundamental organization. In appearance the derived organization changes from time to time either as enduring modifications (Jollos, 1913) or as mutations. In short, the history of the fundamental organization is a history of ceaseless change, but—"*plus ça change plus ça reste la même chose.*"

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FURTHER OBSERVATIONS ON THE DIURNAL MIGRATION OF COPEPODS IN THE GULF OF MAINE

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The following observations were undertaken to obtain further information on the diurnal migration of plankton in relation to environmental conditions. It was felt that the influence of temperature should be more carefully tested, particularly at the thermocline. The possibility that diurnal migration might be caused or controlled by reactions to food organisms required investigation. More precise and more extensive observations were desired on the effect of the changes in submarine irradiation with depth and with time of day. The observations were made at two stations in the Gulf of Maine, the first (Station 1722) occupied for 54 hours on July 14–16, 1933, in the deep central part of the Gulf but farther off shore than the stations of the previous year (Clarke, 1933), and the second (Station 1723) occupied for 24 hours on July 17–18, 1933, on the eastern edge of Georges Bank. By comparing the behavior of the copepods at these different localities, it was believed that the importance of differences both in environmental factors and in physiological condition of the animals would be revealed.

METHODS

The same methods were used for studying the migration as before with the following modifications. The nets were provided with new brass buckets in which the monel metal netting was coarser (18 strands/cm.), thus allowing faster drainage and minimizing clogging. The strap (15, Fig. 1*A*, Clarke, 1933) used to hold the tail of each net at the edge of its frame preliminary to lowering was replaced by a light brass chain through an appropriate link in which the pin (14) was inserted. The pursing rope (*P*) and the cord (18) were provided with spring clips for fastening them to the upper clamp (2) and the handline (19) respectively, thus saving the time formerly consumed in tying knots. To aid in keeping the ship in the same body of water throughout the whole period of observation, a buoy was constructed consisting of a keg with a pole thrust through it at right angles to its long axis. Since our intention was to study the plankton in the upper 50 meters of water particularly, a sea anchor was attached below the keg at a depth of 25 meters. The buoy therefore tended to stay with the body of water at that average depth, and since the keg floated three-quarters submerged, it had very little windage. The buoy was equipped with a flag during the day and with two hurricane lanterns at night and could be seen easily for several miles. The depth of the hauls is indicated in the tables. At the deep station the copepods migrated to such a depth that three series of hauls were sometimes necessary. The qualitative and quantitative analysis of the catch and the length measurements were very kindly carried out for me by Mr. Charles Wheeler assisted by Mr. David Lillie.

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[1] Contribution No. 48.

General Vertical Distribution

The catches were composed almost entirely of *Calanus finmarchicus* (Gunner). A small number of *Metridia lucens* (Boeck)—mostly adult females—was taken also in some cases (Tables I and II). At the deep station *Calanus* copepodid stage V was the most numerous group present. This group, together with copepodid stage IV and the adults of both sexes, were represented at some time of the day at every depth down to 174 meters. Very few individuals in copepodid stage III occurred at this locality. *Metridia* was generally confined to the deeper levels, although at night the range of the adult females and the immature did extend upwards to 6 meters. Since the water was 260 meters deep at this station, there was a considerable depth below the lowest haul which was not sampled at all. At the Bank station the adult females and copepodid stage V of *Calanus* were the most numerous group, but the adult males and stages IV and III were also well represented. All the *Calanus* groups were found at all levels. *Metridia* occurred chiefly at the deeper levels. Since bottom was at 65 to 75 meters at this station, the whole depth of water was sampled in this case.

To aid in distinguishing seasonal broods the cephalothorax length of copepods in the various sex and age groups were measured from representative hauls made at different depths and at different hours during the two stations (Table III). From Table IV it is seen that the ratio of the total length to the cephalothorax length of *Calanus* is extraordinarily constant. This means that relative shape remains the same in spite of differences in size.

The cephalothorax length of *Calanus* adult male changes very little with depth or with time of day. In the case of the adult females considerable constancy of length exists for all the hauls within each station, but at the deep station the averages range from 2.654 mm. to 2.722 mm., whereas at the Bank station the range is from 2.536 mm. to 2.674 mm. If this difference be regarded as significant, it may indicate that we are dealing with two different broods (*cf.* Russell, 1928, 1934; Marshall, 1933). None of the averages for the adult females is as low as that found for the 6-meter level previously, namely, 2.445 mm. (Clarke, 1933, Table VI). Furthermore, in the present case the range is not as great and there is not a distinct increase in length with depth. The average lengths of *Calanus* copepodid stage V show a little more variation at each station, but there is no consistent change with depth and no clear-cut difference between day and night hauls. The range of lengths is about the same at the two stations, and the average size is slightly greater than that found the previous year. Average lengths for copepodid stage IV range from 1.853 mm. to 1.903 mm. at the deep station and from 1.743 mm. to 1.867 mm. at the Bank station. The animals taken this season were generally larger than those of the same stage taken previously. The lengths of 25 specimens of *Calanus* copepodid stage III (Haul 420) ranged from 1.275 to 1.488 mm. and the average, 1.403 mm., is greater than that found the year before. For *Metridia lucens* the range for 27 specimens of the adult females from Haul 342 is 1.828 to 2.253 mm. and the average, 2.014 mm., falls within the range reported previously. In general the length measurements of all the copepods vary much less than was the case the year before and there are no regular changes within each group.

Environmental Conditions

The intensity of light present at different depths and its changes during the course of the day were measured at both the stations (Clarke and Oster, 1934). For the sections of the spectrum considered, red light had very nearly the same intensity as blue at the surface of the water but its intensity diminished very much more rapidly with depth. The intensity of red light was reduced to 1 per cent of its value at the surface at a depth of 11 to 12 meters whereas blue light did not suffer a corresponding reduction until a depth of 26 to 40 meters was reached. Since the copepods appear to have migrated downwards more than 100 meters at the deep station, it seems probable that in so far as their movement is a response to light, it is the blue component rather than the red which is chiefly concerned. Lines of equal intensity for blue light only have been represented in all the figures of diurnal migration.

At the deep station the temperature of the water was measured every 5 meters to a depth of 50 meters and the series was repeated 19 times

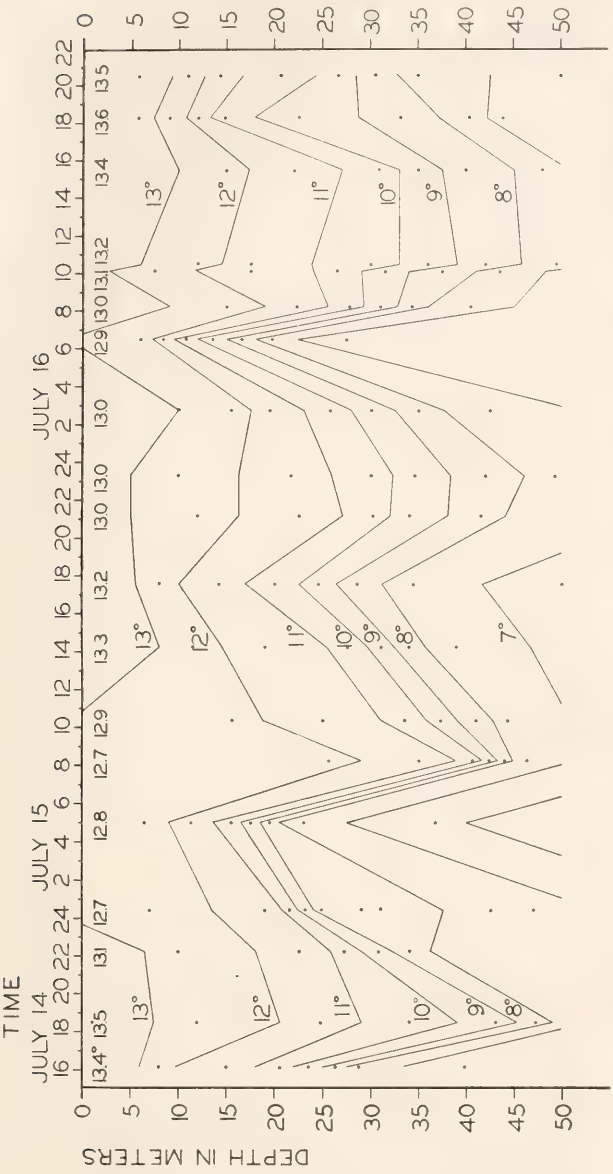

Fig. 1. Variation in temperature down to 50 meters at the deep station (Station 1722). Nineteen series of measurements were made at the hours indicated. The black dots indicate the half-degree points. The temperatures at the top are for the 1-meter level.

during the 54-hour period[2] (see Fig. 1). The isotherms show two sharp peaks: one at 5 o'clock, July 15, and the other at 6 o'clock, July 16. There are three less prominent peaks alternating with these.[3] The diurnal heating effect of the sun can be seen in the variation of the temperature at 1 meter. The difference between the highest and lowest temperatures at this depth was only 0.9° C. At a depth of 28 meters a change of 5° C. occurred in the 3 hours between 5 and 8 o'clock on July 15. Since it would be impossible for a large body of water to change its temperature so quickly, the effect must have been produced by a vertical movement of whole strata of water. This is substantiated by the fact that the thermocline is seen to have been periodically displaced. For example, during the 3 hours following 5 o'clock on July 15 it is moved downwards 25 meters. The temperatures below 50 meters at the deep station (Table I) were measured at three different times and found to vary on the average by about half a degree. For the one temperature observation taken at the Bank station the thermocline occurred between 15 and 20 meters. Data showing variation with depth of oxygen, phosphate, nitrate, and salinity for these stations are given by Braarud (1934, Tables I and II).

The vertical distribution of the phytoplankton at both stations has been reported by Braarud (1934). The phytoplankton was generally poor in both localities, but coccolithophorides, peridinians and ciliates were found above the thermocline and diatoms existed below it. At the deep station *Pontosphæra Huxleyi* was the dominant form and occurred in maximum numbers at 18 meters. Although this species was also the most numerous at the Bank station (maximum abundance at 15 meters), the dominant form in respect to bulk was the much larger *Guinardia flaccida* with a level of maximum abundance at 30 meters.

Diurnal Migration

The sex and age groups of *Calanus finmarchicus* all exhibited much the same type of diurnal migration at the deep station as may be seen from Figs. 2 and 3. The copepods of all groups were distributed chiefly below 80 meters at 1730 (Local sun time) on July 14. At 1930 there was an indication of an upward movement, but at 2130 maximum numbers occurred at the bottom of the series. Probably a rapid upward migration took place during the interval between these two series, with the result that at 2130 the major part of the migrating population was present above 70 meters where no hauls were made. Support for this suggestion is furnished by the fact that copepods occurred in numbers in the upper strata at 20 o'clock on the following two evenings. Furthermore, since a large population existed near the surface at dawn on July 15, an upward migration must have taken place at some time during the night. The descent the following morning was very rapid, for at 0530 the maximum occurred at 66 meters or below and at 9 o'clock the entire population had sunk below 100 meters. At 1630 the upward movement had begun and proceeded rapidly until about 20 o'clock. Not much change in distribution took place during the remainder of the night, but in every case except that of the adult females, the population occurred at a slightly lower level before dawn the next morning (*cf.* Worthington, 1931; Nicholls, 1933). The migration on July 16 followed very closely the course run on the previous day. The indication of a concentration of adult males at the 6-meter level at 0430 on July 16 is probably due to a sampling error, the great majority of animals undoubtedly being below 54 meters at that time (*cf.* Table I). The general nature of the migration of *Calanus* at this station is similar to that observed by Nicholls (1933) for the adult females in the Clyde Sea-Area.

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[2] In connection with meteorological investigations of Prof. C. G. Rossby and Mr. R. T. Montgomery.

[3] Similar fluctuations in temperature were observed by Prof. C. G. Rossby and Prof. A. C. Redfield on June 1, 1934 from the "Atlantis" while anchored at Station 2212 (not yet reported

The slight differences in the behavior of the sex and age groups are chiefly matters of the distance through which the population moved. Thus, although all groups migrated to a depth of 120 meters or more during the day time, the adults descended farther than the immature on July 15. At night, the range of every group extended up to 6 meters but the level of maximum abundance was never higher than 42 meters for the males and never higher than 30 meters for copepodid stages V and IV, whereas for the females it occurred at 6 meters in the first series on July 16. Generally throughout the day the males were distributed deeper than the other groups.

A consistent though rough correlation between the vertical distribution of the copepods and the intensity of the submarine irradiation is apparent from the figures. Practically no individuals were taken from zones where the light intensity was 100 μw or greater. Early in the morning the center of the population coincides fairly closely with the 1.0 μw line, but in the next few hours the copepods descend more rapidly than the lines of equal intensity do, until at 9 o'clock maximum numbers occur at depths where the irradiation is less than 0.01 μw. It appears that insofar as the animals are reacting to light a higher intensity is required to initiate the migration than is required to cause it to continue. At 16 o'clock and thereafter the copepods were found in strata where

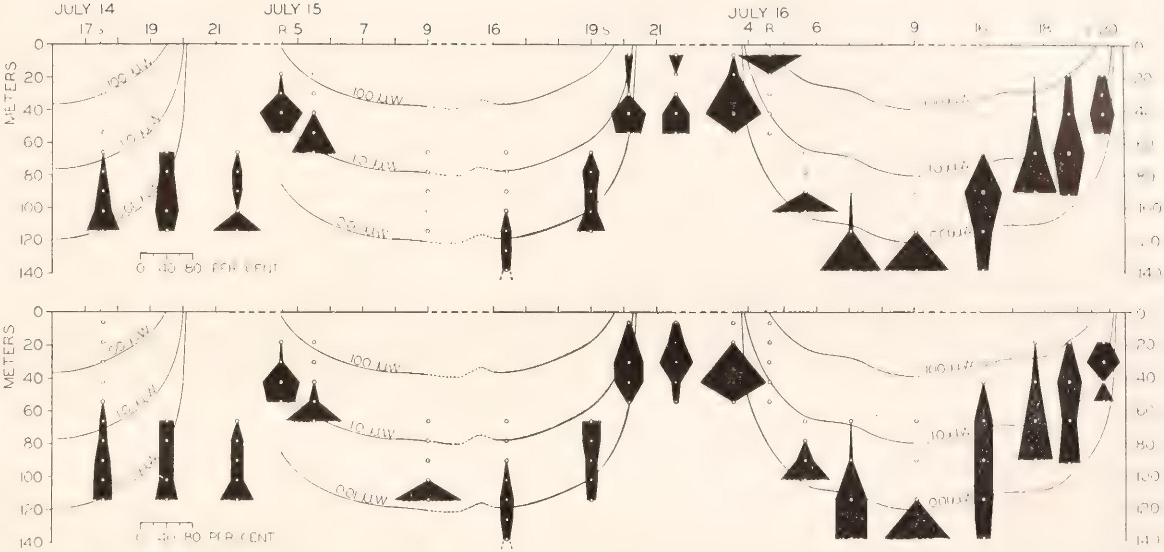

FIG. 2. Upper—*Calanus finmarchicus*, adult male. Lower—*Calanus finmarchicus*, copepodid Stage IV.

The diurnal migration during the 54-hour period at the deep station (Station 1722). The black circles and white dots indicate the depths at which hauls were made. The changes in the intensity of the blue component of daylight are shown by the lines representing the depths at which 100 microwatts/cm.^{2}, 1.0 microwatts/cm.^{2}, etc. occurred. Where the lines are broken, a shortening of the time scale is indicated merely; observations were not discontinued during these periods. *S* = Sunset. *R* = Sunrise.

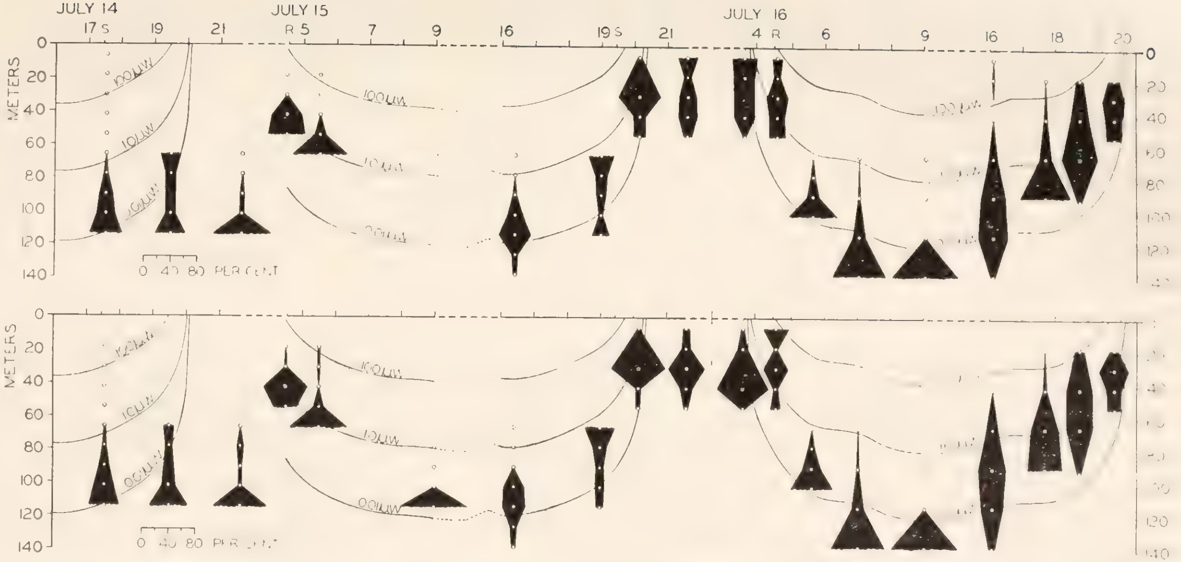

FIG. 3. Upper—*Calanus finmarchicus*, adult female. Lower—*Calanus finmarchicus*, copepodid stage V. The diurnal migration at the deep station (Station 1722).

the irradiation was more than 0.01 μw and in some cases even more than 1.0 μw. This tolerance of higher irradiation in the latter half of the day can be explained as the result of decreased sensitivity following light adaptation (*cf.* Worthington, 1931; Russell, 1931). *Calanus* copepodid stage III was almost entirely absent at the deep station.

Males, females, and immature of *Metridia lucens* occurred in the deep hauls and in the shallower hauls made at night. Toward the end of the afternoon on July 15 *Metridia* was encountered from 138 to 174 meters, and on July 16 from 90 to 138 meters. Females and immature reached the 6-meter level and males reached the 42-meter level at 2138 on July 15, but these strata were deserted early in the morning.

The observations made on the succeeding days at the Bank station revealed an entirely different vertical distribution and diurnal migration of *Calanus,* the major portion of the population being confined to the upper strata at all times (*cf.* Nicholls, 1933, p. 155; Russell, 1934). This station was about 100 miles south of the preceding and located in a physiographically distinct part of the Gulf, but as described above, the environmental conditions were not profoundly different. The sex and age groups of *Calanus* all exhibited the same general behavior (Table II and Figs. 4 to 6). At 16 o'clock on July 17 maximum numbers occurred at depths of from 5 meters to 25 meters, and as night came on there was a slight movement upwards. At 3 o'clock the next morning most groups were rather evenly distributed in the upper 30 meters but an hour later there was indication of a concentration toward the surface again (*cf.* Russell, 1926). Soon after 6 o'clock the main downward migration began. The upper strata were progressively deserted during the remainder of the morning until at 14 o'clock maximum abundance occurred at a depth of 25 meters. By 1530 the copepods had started to move toward the surface again.

The adult males (Fig. 4, upper) did not migrate toward the surface as strongly as did the other groups of *Calanus.* The males were rather evenly distributed throughout the day and the maximum was usually to be found at 12 to 15 meters. The adult females (Fig. 4, lower) not only were much more abundant in the uppermost water layer but also were taken in even smaller numbers below 30 meters. This group exhibited little tendency toward uniform distribution during the night and did not leave the surface as early in the morning as did the males. The copepodid stage V population (Fig. 5, upper) was not highly concentrated, being well represented at all depths even down to 65 meters, but its vertical movement was slightly more extensive than that of the adults. Copepodid stage IV (Fig. 5, lower) migrated more strongly toward the

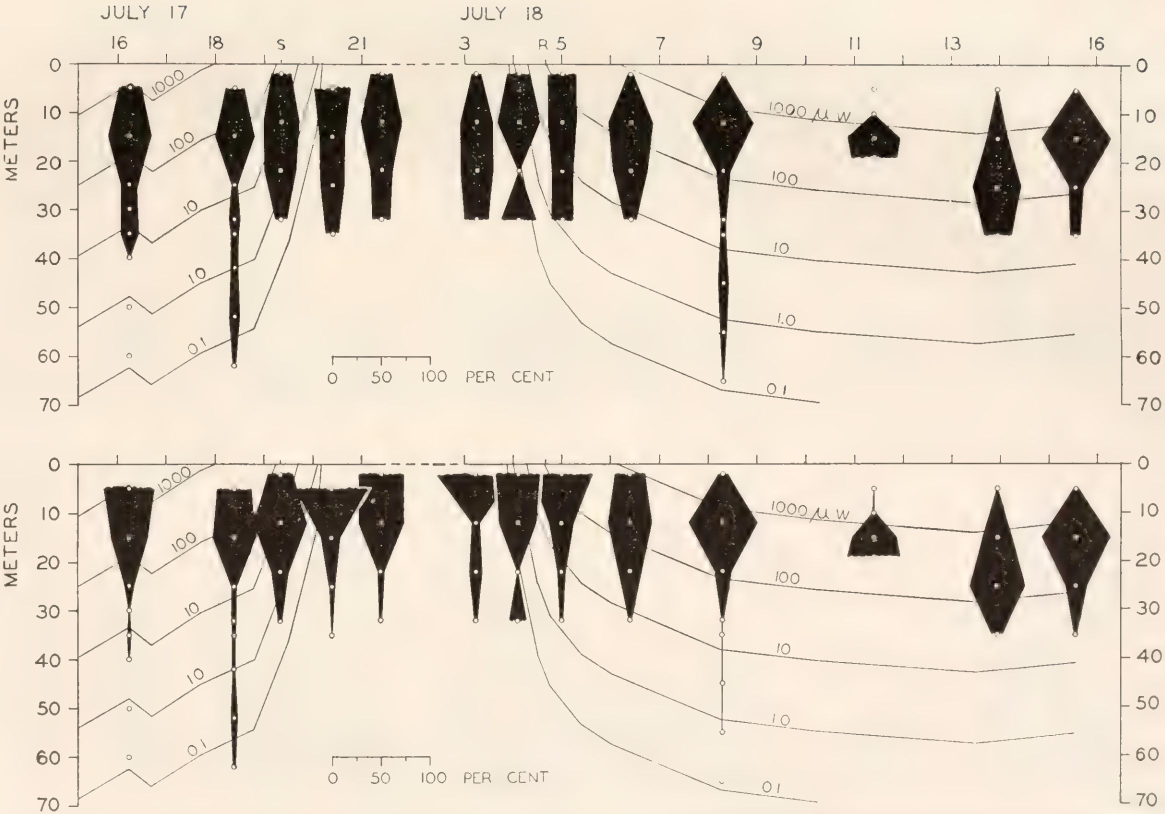

FIG. 4. Upper—*Calanus finmarchicus,* adult male. Lower—*Calanus finmarchicus,* adult female. The diurnal migration during the 24-hour period at the Bank station (Station 1723).

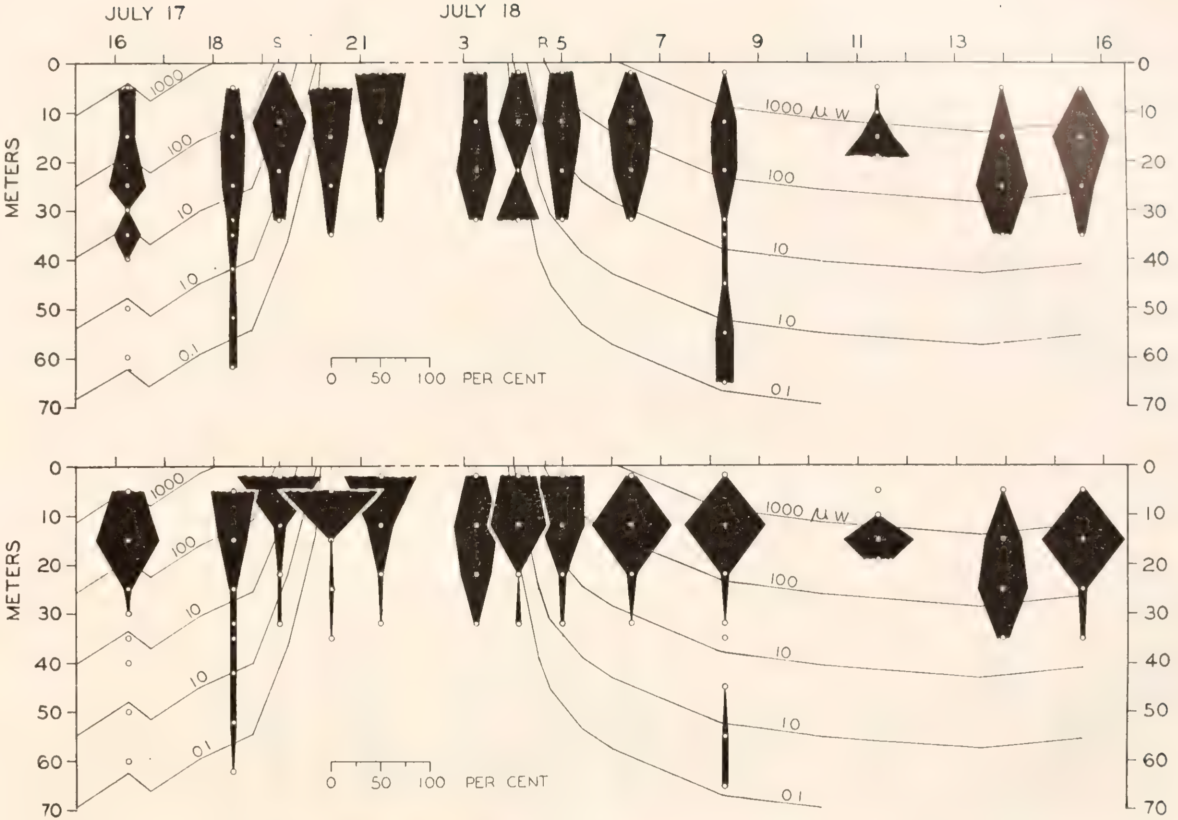

Fig. 5. Upper—*Calanus finmarchicus*, copepodid stage V. Lower—*Calanus finmarchicus*, copepodid stage IV. The diurnal migration at the Bank station (Station 1723).

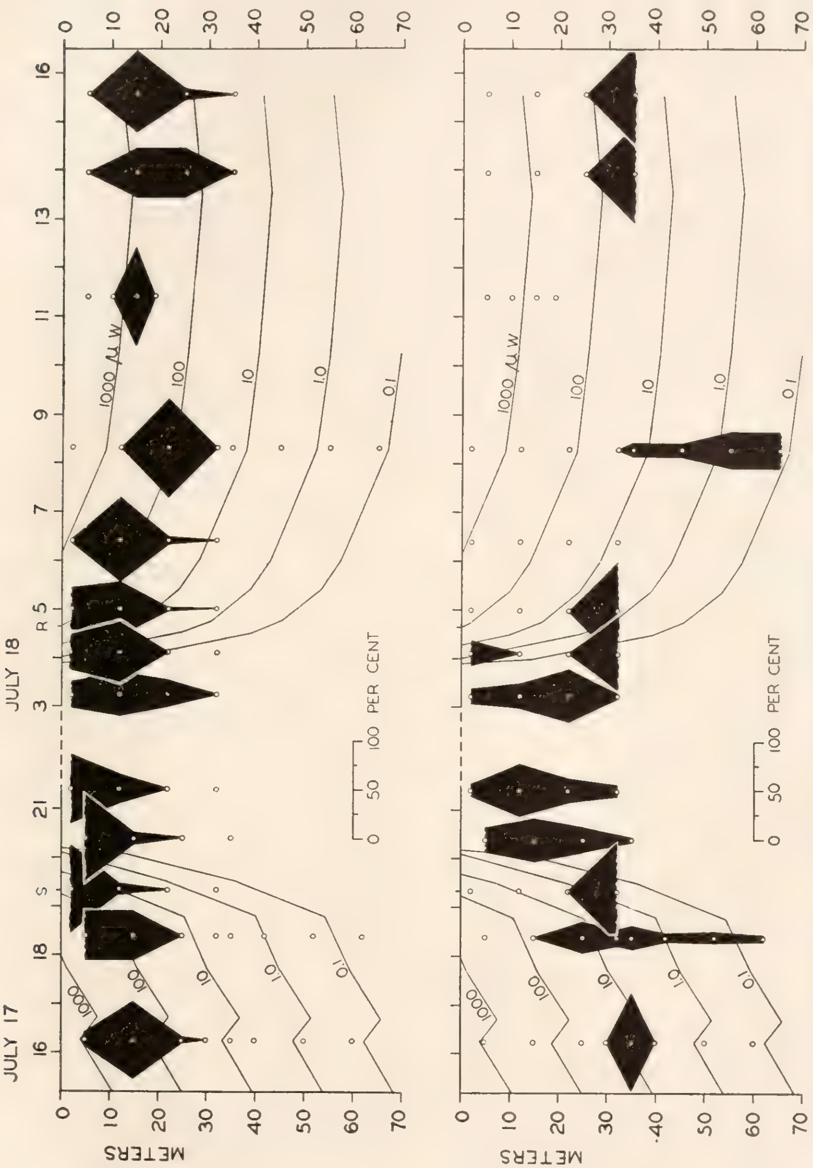

FIG. 6. Upper—*Calanus finmarchicus*, copepodid stage III. Lower—*Metridia lucens*, adult female. The diurnal migration at the Bank station (Station 1723).

surface at night than stage V and did not descend as far during the day. The tendency toward uniform distribution early in the morning is clearly marked. The distribution of copepodid stage III (Fig. 6, upper) was much the same as stage IV except that the level of maximum abundance never occurred below 22 meters and extremely few individuals were taken below 25 meters at any time.

While the *Calanus* groups were carrying out these very limited migrations at this station, the *Metridia* population showed a considerable vertical movement. During the first afternoon and evening the level of maximum abundance of the adult females (Fig. 6, lower) moved upwards from 35 meters to 12 meters. The animals did not continue migrating toward the surface during the night as previously (Clarke, 1933), for early the next morning the maximum occurred at 22 meters. A rapid downward movement followed until at 0830 the population was found chiefly below 35 meters. The adult males and immature of *Metridia,* which were taken in small numbers, appear to have migrated in much the same manner as the adult females, although not as extensively.

Since all the groups of *Calanus* remained in the upper water layers throughout the day at this station the animals must have been able to withstand strong irradiation. It can be seen from the figures that the copepods sometimes occurred in large numbers at levels where the light intensity was 1000 μw or more. However, the maximum abundance for all the groups of *Calanus* was usually found between the 1000 μw and the 100 μw lines. In most cases it is evident that the animals are inhabiting zones of higher light intensity in the afternoon than early in the morning, indicating that light-adaptation has taken place. The adult females of *Metridia* were confined to zones of lower irradiation. In the morning they appear to have avoided light intensities higher than 10 μw, but later in the day they were taken at levels where the irradiation was between 100 μw and 10 μw.

Discussion

The present observations illustrate the fact frequently pointed out by other investigators that the diurnal migration of plankton is a highly variable affair (Russell, 1932; Southern and Gardiner, 1932). The fact that the sex and age groups of *Calanus finmarchicus* all exhibited much the same type of behavior in the present case is in sharp contrast with the observations of Nicholls (1933) on this species made during his excellent investigations in the Clyde Sea-Area. At our deep station *Calanus* was found to be carrying out an extensive migration but at our

Bank station the same species exhibited a very slight diurnal movement. At the deep station the temperature was generally lower than at the Bank station, there being about 2° C. difference at the surface. Since *Calanus* is a cold-water species one would have expected a movement away from the surface in the warmer locality rather than in the colder. At the deep station the copepods carried out their migration in spite of the vertical oscillations of the water strata. The fact that the animals moved across the thermocline shows that in this case, at least, temperature is of minor importance in controlling vertical distribution (*cf.* Clarke, 1934*b*; Russell, 1927, p. 241). At the Bank station the copepods remained in large measure above the thermocline.

The salinity of the upper strata was slightly lower at the deep station than at the Bank station, but below 30 meters it was much the same at both localities. There was very little difference in other hydrographic conditions at the two stations (Braarud, 1934) and the differences in the phytoplankton do not appear to be significant although we do not know precisely which forms are important as food organisms (*cf.* Clarke, 1934*a*). The dominant form, *Pontosphæra,* was abundant above the thermocline in both cases, and the diatom population below the thermocline at the Bank station can be of no importance since the majority of the animals did not visit the lower strata. In view of this situation not only does it appear that the differences in the behavior at the two stations are not due to differences in the phytoplankton, but also it is difficult to understand how food could be an important factor in causing or controlling the diurnal migration which did occur at the deep station (*cf.* Worthington, 1931; Nicholls, 1933). In their upward movement the animals passed right through the zone of abundance of phytoplankton.

The year before in the Gulf of Maine at Stations 1285 and 1286 (Clarke, 1933) the adult females of *Calanus* exhibited no regular movement and copepodid stages V and IV migrated over a very limited range only. At Station 1287 the adult female population tended to rise and fall between 16 and 30 meters, but the copepodid stages showed very little tendency to migrate. In general in both cases the females and stages V and IV occurred in nearly equal numbers at all depths. The environmental conditions at the time when these observations were made were not strikingly dissimilar from those at Stations 1722 and 1723.

Metridia carried out distinct migrations at both Stations 1722 and 1723, although in the former case the vertical movement was more extensive. The species—particularly the adult females—is evidently more sensitive to light than *Calanus* since it was confined to zones of less intense irradiation. Furthermore *Metridia* arrived at the surface later than *Calanus* and left the surface earlier at both stations. The migration of the adult females of *Metridia* was generally similar to that observed for this group the year before at Station 1287 but differed in certain details. At Stations 1722 and 1723 the copepods did not show a tendency to continue swimming toward the surface after complete darkness as they did on the previous occasion. Moreover, in the present case the downward migration was begun more than an hour before sunrise, and the animals descended during the day to much greater depths at Station 1722 than they did at Station 1287 where the water was correspondingly deep.

These differences in behavior must represent differences in the physiological condition of the organisms. In the case of the wide disparity in the distribution and migration of *Calanus* at the deep station and the Bank station, the conclusion seems inevitable that a fundamental difference in the reaction of the animals to light existed. It may be that we are dealing with two distinct broods, which differed in their physiological states (*cf.* Russell, 1928, 1934), although no clear indication of this appears from the length measurements. On the other hand, dissimilarities in the response to light might have been caused within comparable broods by the differences in the environmental conditions even though these are apparently slight. It is significant to note, however, that nothing in the environment at the Bank station prevented *Metridia* from carrying out a considerable migration. Although it is well known that differences in the environment often affect the reactions of different organisms in diverse ways, the present state of our knowledge of the specific action of external factors on copepods is insufficient to explain the variations in these observations on diurnal migration. However, we do know that phototropic reactions are sometimes under a very delicate control (Clarke, 1932).

We may conclude that in the control of the diurnal migration of copepods the environmental conditions are important from two points of view. First, they have a direct action in providing the stimuli by which the animals' swimming reactions may be evoked. Secondly, an indirect action is involved by which the factors of the environment influence the physiological condition of the organism. The environment is responsible for the physiological differences which may exist not only between seasonal broods but also between individuals of the same brood. Of the factors which directly affect the vertical movement of the copepods, light is the most important, but the migration may be indirectly influenced by the environment if conditions are such that the animals' reactions to light and to the other factors are changed.

SUMMARY

1. Observations were made on the diurnal migration of copepods in relation to submarine irradiation and other external factors at a 54-hour and a 24-hour station in the Gulf of Maine using the same methods as previously. The zoöplankton consisted almost entirely of *Calanus finmarchicus* with a small number of *Metridia lucens*. The length of the copepods varied much less than was the case previously.

2. The variation with depth of the light intensity, the temperature, chemical constituents, and the phytoplankton was observed. During the first station the temperature was found to fluctuate widely.

3. At the station in the deep part of the Gulf all groups of *Calanus* and *Metridia* migrated to a depth of 120 meters or more during the daytime and moved upwards to levels of from 6 meters to 42 meters at night. At the station on Georges Bank *Calanus* was confined to the surface strata, undergoing only a very limited migration, but *Metridia* carried out an extensive migration.

4. The migration of the copepods was found to be more closely correlated with the changes in the submarine irradiation than with changes in the hydrographic conditions or in the phytoplankton. However, great variability in the behavior was observed. This appears to be due to differences in the physiological condition of the animals and represents the indirect action of the environment.

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Table I

Station 1722, Lat. 42° 50′ N., Long. 67° 05′ W.

Hauls *A*301–*A*320: July 14, 1933; sea: slight; sky: overcast; sunset: 1927.

Hauls *A*322–*A*360: July 15, 1933; sea: slight; sky: few clouds, fog; sunrise: 0433; sunset: 1926.

Hauls *A*361–*A*403: July 16, 1933; sea: slight becoming smooth; sky: overcast; sunrise: 0434; sunset: 1926.

Temperature: (See also Fig. 1) 54 meters, 6.50° C.; 72 meters, 6.25° C.; 90 meters, 5.57° C.; 113 meters, 5.74° C.; 136 meters, 6.01° C.; 158 meters, 6.53° C.; 181 meters, 6.90° C.; 202 meters, 7.06° C.; 227 meters, 7.02° C.

Numbers of copepods in hundreds; $x =$ less than 50.

The data for each haul are given in the following order: haul number, depth (meters), local sun time; irradiation at surface (μw/cm.^{2}), irradiation at haul depth (μw/cm.^{2}), irradiation 30 minutes before (%): *Calanus finmarchicus* adult male, adult female, cop. V, cop. IV, cop. III; *Metridia lucens* adult male, adult female, immature.

*A*301, 6, 1706; 4000, 1900, 140: x, x, x, x, 0; 0, 0, 0
*A*302, 18, 1706; 4000, 390, 140: x, x, x, x, 0; 0, 0, 0
*A*303, 30, 1706; 4000, 110, 140: x, x, x, x, 0; 0, 0, 0
*A*304, 42, 1706; 4000, 34, 140: x, x, x, x, 0; 0, 0, 0
*A*305, 54, 1706; 4000, 8.4, 140: x, x, x, x, 0; 0, 0, 0
*A*306, 66, 1759; 1700, 1.0, 170: x, x, 3, 2, 0; 0, 0, 0
*A*307, 78, 1759; 1700, —, 170: 1, 5, 17, 3, 0; 0, 0, 0
*A*308, 90, 1759; 1700, —, 170: 2, 15, 46, 5, 0; 0, 0, 0
*A*309, 102, 1759; 1700, —, 170: 3, 22, 90, 6, 0; 0, 0, 0
*A*310, 114, 1759; 1700, —, 170: 6, 39, 141, 6, 0; 0, 0, 0
*A*311, 66, 1936; 44, —, 770: 3, 2, 3, 2, 0; 3, 15, 6
*A*312, 78, 1936; 44, —, 770: 3, 1, 2, 2, x; 3, 11, 10
*A*314, 102, 1936; 44, —, 770: 5, 1, 4, 2, 0; 7, 29, 3
*A*315, 114, 1936; 44, —, 770: 3, 3, 12, 3, x; 2, 10, 19
*A*316, 66, 2139; 0, 0, no light: x, x, x, x, 0; x, x, x
*A*317, 78, 2139; 0, 0, no light: 1, x, 6, 1, 0; 1, 1, 2
*A*318, 90, 2139; 0, 0, no light; 1, 1, 3, 1, 0; 1, 5, 2
*A*319, 102, 2139; 0, 0, no light: x, 1, 3, 1, 0; x, x, 1
*A*320, 114, 2139; 0, 0, no light: 5, 14, 50, 3, 0; 3, 8, 9
*A*322, 18, 0429; 140, 14, 6: x, x, x, 0, 0; 0, x, 0
*A*323, 30, 0429; 140, 3.8, 6: 1, 1, 7, 1, 0; 0, 0, 0
*A*324, 42, 0429; 140, 0.9, 6: 7, 66, 697, 24, 0; 0, 0, 0
*A*325, 54, 0429; 140, 0.3, 6: 3, 79, 366, 17, 0; 0, 0, 0
*A*326, 18, 0531; 1300, 130, 46: x, x, 1, x, 0; 0, 0, 0
*A*327, 30, 0531; 1300, 35, 46: x, x, 1, x, 0; 0, 0, 0
*A*328, 42, 0531; 1300, 11, 46: 0, 0, 0, 0, 0; 0, 0, 0
*A*329, 54, 0531; 1300, 2.7, 46: 1, 2, 24, 1, 0; 0, 0, 0
*A*330, 66, 0531; 1300, 0.7, 46: 2, 10, 137, 6, 0; 0, 0, 0
*A*331, 66, 0859; 7100, 4.0, 86: 0, 0, x, x, 0; 0, 0, 0
*A*332, 78, 0859; 7100, 1.1, 86: x, x, x, x, 0; 0, 0, 0
*A*333, 90, 0859; 7100, —, 86: 0, x, x, x, 0; 0, 0, 0
*A*334, 102, 0859; 7100, —, 86: 0, x, x, x, 0; 0, 0, 0
*A*335, 114, 0859; 7100, —, 86: x, x, 3, 1, 0; 0, 0, 0
*A*336, 66, 1558; 6200, 3.5, 130: 0, x, x, x, 0; 0, 0, 0
*A*337, 78, 1558; 6200, 0.9, 130: 0, x, x, x, 0; 0, 0, 0
*A*338, 90, 1558; 6200, 0.2, 130: x, 4, 24, x, 0; 0, 0, 0
*A*339, 102, 1558; 6200, —, 130: 0, 53, 263, 3, 0; 0, 0, 0
*A*340, 114, 1558; 6200, —, 130: 3, 121, 283, 7, 0; 0, 0, 0
*A*341, 126, 1652; 4700, —, 140: 3, 24, 56, 6, 0; 0, 0, 0
*A*342, 138, 1652; 4700, —, 140: 2, 10, 44, 3, 0; 1, 7, 10

TABLE I—*Continued*

*A*343, 150, 1652; 4700, —, 140: 3, 22, 47, 3, 0; 2, 7, 5
*A*344, 162, 1652; 4700, —, 140: 7, 10, 41, 6, 0; 4, 7, 4
*A*345, 174, 1652; 4700, —, 140: 3, 14, 93, 7, 0; 5, 3, 10
*A*346, 66, 1858; 810, 0.5, 150: x, 117, 1079, 23, 0; 0, 0, 0
*A*347, 78, 1858; 810, —, 150: 4, 57, 486, 23, 0; 0, 0, 4
*A*348, 90, 1858; 810, —, 150: 3, 41, 251, 14, 0; 0, 0, 0
*A*349, 102, 1858; 810, —, 150: 3, 17, 283, 14, 0; 0, 0, 3
*A*350, 114, 1858; 810, —, 150: 7, 83, 258, 10, 0; 3, 3, 17
*A*351, 6, 2009; 3.9, 1.8, 610: 2, 23, 76, 2, 0; 0, 0, 0
*A*353, 30, 2009; 3.9, 0.1, 610: x, 375, 2580, 42, 0; 0, 0, 0
*A*354, 42, 2009; 3.9, —, 610: 10, 104, 262, 41, 0; 0, 35, 10
*A*355, 54, 2009; 3.9, —, 610: 7, 104, 217, 10, 0; 3, 210, 35
*A*356, 6, 2138; 0, 0, no light: 4, 164, 312, 11, 0; 0, 8, 4
*A*357, 18, 2138; 0, 0, no light: 0, 61, 410, 27, 0; 0, 4, 0
*A*358, 30, 2138; 0, 0, no light: x, 150, 1855, 60, 0; 0, 0, 0
*A*359, 42, 2138; 0, 0, no light: 8, 163, 627, 15, 0; 4, 76, 53
*A*360, 54, 2138; 0, 0, no light: 8, 68, 213, 8, 0; 4, 125, 38
*A*361, 6, 0342; 0, 0, no light: x, 126, 14, 0, 0; 0, 2, 0
*A*362, 18, 0342; 0, 0, no light: 2, 111, 109, 0, 0; 0, 0, 0
*A*364, 42, 0342; 0, 0, no light: 11, 110, 567, 8, 0; 0, 4, 4
*A*365, 54, 0342; 0, 0, no light: x, 6, 32, x, 0; 0, 0, 0
*A*366, 6, 0434; 78, 39, 3: 1, 12, 25, 0, 0; 0, 0, 0
*A*367, 18, 0434; 78, 7.8, 3: x, 7, 3, x, 0; 0, 0, 0
*A*368, 30, 0434; 78, 1.2, 3: x, 28, 25, x, 0; 0, 0, 0
*A*369, 42, 0434; 78, 0.3, 3: x, 19, 5, 0, 0; 0, 0, 0
*A*370, 54, 0434; 78, —, 3: x, 21, 9, x, 0; 0, 0, 0
*A*372, 66, 0539; 1300, 0.7, 26: 0, x, x, x, 0; 0, 0, 0
*A*373, 78, 0539; 1300, —, 26: x, 2, 6, x, 0; 0, 0, 0
*A*374, 90, 0539; 1300, —, 26: x, 5, 17, 1, 0; 0, 0, 0
*A*375, 102, 0539; 1300, —, 26: 1, 19, 39, 3, 0; 0, 0, 0
*A*376, 66, 0702; 2800, 1.6, 61: 0, x, 1, x, 0; 0, 0, 0
*A*377, 90, 0702; 2800, 0.1, 61: x, 1, 4, 1, 0; 0, 0, 0
*A*378, 114, 0702; 2800, —, 61: 1, 17, 83, 11, 0; 0, 0, 0
*A*379, 138, 0702; 2800, —, 61: 15, 72, 349, 11, 0; 0, 4, 15
*A*380, 66, 0902; 7500, 4.3, 87: x, x, x, x, 0; 0, 0, 0
*A*381, 90, 0902; 7500, 0.3, 87: x, x, x, x, 0; 0, 0, 0
*A*382, 114, 0902; 7500, —, 87: 0, x, x, x, 0; x, 0, x
*A*383, 138, 0902; 7500, —, 87: 4, 4, 95, 23, 0; 6, 2, 21
*A*384, 6, 1547; 3900, 2000, 110: x, x, x, 0; 0, 0, 0
*A*385, 18, 1547; 3900, 390, 110: x, 2, 1, x, 0; 0, 0, 0
*A*386, 30, 1547; 3900, 59, 110: x, x, x, x, 0; 0, 0, 0
*A*387, 42, 1547; 3900, 15, 110: x, x, x, x, x; 0, 0, 0
*A*388, 66, 1628; 2800, 1.6, 130; 0, 48, 149, 4, 0; 0, 0, 0
*A*389, 90, 1628; 2800, 0.1, 130: 8, 144, 353, 4, 0; 0, 4, 8
*A*390, 114, 1628; 2800, —, 130: 4, 171, 281, 4, 0; 4, 4, 4
*A*391, 138, 1628; 2800, —, 130: 2, 14, 73, 3, 0; 2, 7, 12
*A*392, 18, 1743; 1200, 120, 200: x, 2, 5, 1, x; 0, 1, x
*A*393, 42, 1743; 1200, 4.6, 200: 3, 14, 47, 4, 0; 0, x, 1
*A*394, 66, 1743; 1200, 0.7, 200: 15, 80, 433, 11, 0; 0, 0, 0
*A*395, 90, 1743; 1200, —, 200: 36, 320, 515, 18, 0; 0, 0, 0
*A*396, 18, 1844; 910, 91, 130: 3, 57, 85, 2, 0; 0, 0, 0
*A*397, 42, 1844; 910, 3.5, 130: 7, 148, 248, 7, 0; 0, 0, 0
*A*398, 66, 1844; 910, 0.5, 130: 19, 281, 247, 4, 0; 0, 4, 11
*A*399, 92, 1844; 910, —, 130: 10, 30, 36, 6, 0; 3, 23, 17
*A*400, 18, 1948; 18, 1.8, 1300: 7, 221, 186, 7, 0; 0, 0, 0
*A*401, 30, 1948; 18, 0.3, 1300: 15, 426, 643, 19, 4; 0, 0, 0
*A*402, 42, 1948; 18, —, 1300: 24, 345, 322, x, 0; 0, x, 12
*A*403, 54, 1948; 18, —, 1300: 15, 274, 293, 11, 0; 0, 42, 19

Table II

Station 1723, Lat. 41° 20′ N., Long. 66° 40′ W.

Hauls *A*404–*A*431, July 17, 1933, Sea: smooth, heavy swell; sky: clear, fog; sunset: 1920.

Hauls *A*432–*A*467, July 18, 1933; sea: smooth, becoming slight, moderate swell; sky: few clouds, fog; sunrise: 0440; sunset: 1919.

Temperature: 1 meter, 15.45° C.; 5 meters, 15.47° C.; 10 meters, 15.39° C.; 15 meters, 13.92° C.; 20 meters, 10.34° C.; 25 meters, 8.67° C.; 30 meters, 8.47° C.; 40 meters, 8.44° C.; 50 meters, 8.21° C.; 60 meters, 8.07° C.; 70 meters, 7.98° C.

Numbers of copepods in hundreds; $x =$ less than 50.

The data for each haul are given in the following order: Haul number, depth (meters), local suntime; irradiation at surface (μw/cm.^{2}), irradiation at haul depth (μw/cm.^{2}), irradiation 30 minutes before (%): *Calanus finmarchicus,* adult male, adult female, cop. V, cop. IV, cop. III; *Metridia lucens,* adult male, adult female, immature.

*A*404, 5, 1558; 3200, 1400, —: 10, 76, 31, 10, 2; 0, 0, 0
*A*405, 15, 1558; 3200, 190, —: 20, 55, 30, 20, 11; 0, 0, 0
*A*406, 25, 1558; 3200, 35, —: 8, 13, 65, 2, 1; 0, 0, 0
*A*407, 35, 1558; 3200, 7.0, —: 8, 4, 47, 0, 0; 4, 35, 5
*A*408, 30, 1632; 3700, 22, 87: x, x, x, x, 0; 0, x, 0
*A*409, 40, 1632; 3700, 4.8, 87: x, x, x, x, x; x, x, x
*A*410, 50, 1632; 3700, 1.0, 87: x, x, x, x, x; 0, 0, 0
*A*411, 60, 1632; 3700, 0.2, 87: x, x, x, x, 0; 0, 0, 0
*A*412, 5, 1759; 1400, 600, 120: 11, 34, 32, 8, 3; 0, 0, 0
*A*413, 15, 1759; 1400, 83, 120: 23, 42, 69, 5, 3; 0, 0, 0
*A*414, 25, 1759; 1400, 15, 120: 3, 4, 58, 1, 0; 0, 14, 1
*A*415, 35, 1759; 1400, 3.1, 120: 4, 2, 31, 1, 0; 0, 10, x
*A*416, 32, 1847; 650, 2.3, 170: 6, 4, 33, 1, 0; 0, 8, 0
*A*417, 42, 1847; 650, 0.7, 170: 4, 2, 14, 1, x; 8, 4, 1
*A*418, 52, 1847; 650, 0.1, 170: 6, 6, 19, 1, 0; 10, 5, x
*A*419, 62, 1847; 650, —, 170: 1, 2, 20, x, 0; 3, 3, 1
*A*420, 2, 1921; 99, 56, 530: 14, 86, 24, 77, 24; 0, 0, 0
*A*421, 12, 1921; 99, 13, 530: 25, 192, 143, 12, 3; 0, 0, 0
*A*422, 22, 1921; 99, 1.7, 530: 22, 61, 58, 1, 0; 0, 0, 0
*A*423, 32, 1921; 99, 0.3, 530: 8, 10, 33, 1, 0; 0, 12, 0
*A*424, 5, 2024; 0, 0, (5.2 μw): 14, 183, 121, 66, 14; 0, 7, 0
*A*425, 15, 2024; 0, 0, (5.2 μw): 10, 47, 99, 0, 2; 0, 12, 0
*A*426, 25, 2024; 0, 0, (5.2 μw): 9, 13, 47, 2, 0; 0, 7, 1
*A*427, 35, 2024; 0, 0, (5.2 μw): 5, 2, 15, x, 0; x, 1, 1
*A*428, 2, 2126; 0, 0, no light: 7, 87, 125, 34, 12; 2, 2, 0
*A*429, 12, 2126; 0, 0, no light: 14, 88, 85, 12, 5; 0, 9, 0
*A*430, 22, 2126; 0, 0, no light: 7, 10, 21, 1, x; 1, 4, 0
*A*431, 32, 2126; 0, 0, no light: 6, 3, 15, x, 0; x, 2, 0
*A*432, 2, 0315; 0, 0, no light: 3, 245, 52, 7, 3; 0, 3, 0
*A*433, 12, 0315; 0, 0, no light: 11, 23, 55, 15, 5; 0, 5, 0
*A*434, 22, 0315; 0, 0, no light: 11, 36, 91, 10, 3; 0, 10, 0
*A*435, 32, 0315; 0, 0, no light: 8, 6, 29, 1, 0; 1, 1, 0
*A*436, 2, 0406; 2.6, 1.5, no light: 3, 32, 7, 7, 5; 0, 1, 0
*A*437, 12, 0406; 2.6, 0.3, no light: 6, 27, 19, 11, 8; 0, 0, 0
*A*438, 22, 0406; 2.6, —, no light: x, 1, 1, x, 0; 0, x, 0
*A*439, 32, 0406; 2.6, —, no light: 5, 12, 20, 1, 0; 1, 3, 1
*A*440, 2, 0501; 380, 210, 16: 4, 75, 13, 31, 20; 0, 0, 0

Table II—*Continued*

*A*441, 12, 0501; 380, 49, 16: 4, 30, 22, 31, 26; 0, 0, 0
*A*442, 22, 0501; 380, 6.5, 16: 3, 10, 15, 5, 1; 0, 0, 0
*A*443, 32, 0501; 380, 1.3, 16: 3, 4, 7, 1, x; x, 1, x
*A*444, 2, 0626; 1600, 900, 81: 1, 26, 8, 3, 1; 0, 0, 0
*A*445, 12, 0626; 1600, 210, 81: 8, 40, 36, 25, 13; 0, 0, 0
*A*446, 22, 0626; 1600, 28, 81: 6, 20, 28, 3, 1; 0, 0, 0
*A*447, 32, 0626; 1600, 5.6, 81: 2, 2, 6, x, x; 0, 0, 0
*A*448, 2, 0802; 4900, 2800, 76: 0, 7, x, x, 0; 0, 0, 0
*A*449, 12, 0802; 4900, 640, 76: 13, 53, 30, 18, 6; 0, 0, 0
*A*450, 22, 0802; 4900, 85, 76: 3, 13, 31, 2, 0; 0, 0, 0
*A*451, 32, 0802; 4900, 17, 76: 1, 1, 5, 0, 0; 0, 0, 0
*A*452, 35, 0836; 6200, 14, 79: 1, x, 6, x, x; 1, 1, x
*A*453, 45, 0836; 6200, 4.0, 79: 2, 1, 5, x, 0; 1, 1, 1
*A*454, 55, 0836; 6200, 0.7, 79: 1, 1, 23, 1, x; 1, 3, 2
*A*455, 65, 0836; 6200, 0.1, 79: x, x, 22, 1, 0; 2, 3, 1
*A*456, 5, 1126; 11,500, 4900, 88: 0, x, x, x, 0; 0, 0, 0
*A*457, 10, 1126; 11,500, 2300, 88: 0, 1, 1, x, 0; 0, 0, 0
*A*458, 15, 1126; 11,500, 680, 88: 25, 60, 130, 15, 12; 0, 0, 0
*A*459, 19, 1126; 11,500, 320, 88: 22, 69, 259, 6, 0; 0, 0, 0
*A*460, 5, 1357; 10,800, 4600, 120: x, x, 0, 0, 0; 0, 0, 0
*A*461, 15, 1357; 10,800, 640, 120: 6, 30, 46, 6, 1; 0, 0, 0
*A*462, 25, 1357; 10,800, 120, 120: 14, 58, 84, 8, 1; 0, 0, 0
*A*463, 35, 1357; 10,800, 24, 120: 8, 11, 30, 2, 0; 0, 1, 0
*A*464, 5, 1535; 9000, 3800, 110: 1, 7, 12, 1, x; 0, 0, 0
*A*465, 15, 1535; 9000, 530, 110: 28, 91, 97, 28, 8; 0, 0, 0
*A*466, 25, 1535; 9000, 100, 110: 6, 28, 44, 3, 1; 0, 0, 0
*A*467, 35, 1535; 9000, 20, 110: 4, 3, 11, 1, x; 0, 1, x

TABLE III

Cephalothorax lengths of Calanus finmarchicus

	Station 1722									Station 1723							
	Day Hauls						Night Hauls			Day Hauls					Night Hauls		
	Haul 384 6 m.	Haul 386 30 m.	Haul 336 66 m.	Haul 339 102 m.	Haul 342 138 m.	Haul 345 174 m.	Haul 356 6 m.	Haul 358 30 m.	Haul 360 54 m.	Haul 404 5 m.	Haul 405 15 m.	Haul 408 30 m.	Haul 409 40 m.	Haul 411 60 m.	Haul 428 2 m.	Haul 429 12 m.	Haul 431 32 m.
Adult male																	
No measured......	—	—	—	—	—	—	—	—	—	25	25	—	9	6	16	24	25
Average length mm.	—	—	—	—	—	—	—	—	—	2.506	2.504	—	2.508	2.416	2.553	2.455	2.514
Range.....	—	—	—	—	—	—	—	—	—	2.295–2.635	2.380–2.720	—	2.423–2.678	2.338–2.508	2.380–2.720	2.253–2.635	2.210–2.720
Adult female																	
No. measured.....	—	—	—	24	25	25	25	25	25	25	25	3	17	26	53	25	26
Average length mm.	—	—	—	2.722	2.710	2.693	2.654	2.715	2.669	2.581	2.606	2.621	2.588	2.536	2.674	2.640	2.621
Range.....	—	—	—	2.465–3.103	2.465–2.975	2.380–3.103	2.465–2.975	2.380–3.018	2.380–3.060	2.210–2.848	2.423–2.805	2.465–2.720	2.380–2.805	2.380–2.720	2.380–3.018	2.465–3.145	2.423–2.933

TABLE III—*Continued*

	Station 1722									Station 1723							
	Day Hauls						Night Hauls			Day Hauls					Night Hauls		
	Haul 384 6 m.	Haul 386 30 m.	Haul 336 66 m.	Haul 339 102 m.	Haul 342 138 m.	Haul 345 174 m.	Haul 356 6 m.	Haul 358 30 m.	Haul 360 54 m.	Haul 404 5 m.	Haul 405 15 m.	Haul 408 30 m.	Haul 409 40 m.	Haul 411 60 m.	Haul 428 2 m.	Haul 429 12 m.	Haul 431 32 m.
Copepodid V																	
No. measured......	5	26	25	50	52	50	50	49	50	50	50	21	11	12	100	51	50
Average length mm.	2.431	2.240	2.220	2.424	2.460	2.399	2.422	2.388	2.436	2.417	2.458	2.374	2.241	2.271	2.434	2.329	2.350
Range.....	2.125– 2.720	2.040– 2.465	2.040– 2.423	2.168– 2.720	2.125– 2.720	2.125– 2.763	2.125– 2.763	2.083– 2.593	2.125 2.720	2.168– 2.720	2.168– 2.635	1.998– 2.593	1.955– 2.423	1.998 2.465	1.998– 2.720	2.040 2.720	1.955 2.635
Copepodid IV																	
No. measured......	—	—	—	—	25	25	25	12	12	25	25	1	1	1	50	26	2
Average length mm.	—	—	—	—	1.903	1.874	1.875	1.885	1.853	1.826	1.867	1.785	1.743	1.785	1.845	1.849	1.807
Range.....	—	—	—	—	1.785– 2.040	1.700– 2.338	1.658– 2.040	1.828– 2.083	1.785– 1.955	1.615– 1.955	1.743– 2.083	—	—	—	1.700– 1.998	1.700– 2.083	1.785– 1.828

TABLE IV

Comparison of Cephalothorax length and total length in Calanus finmarchicus

	Haul No.	No. measured	Average cephalothorax length	Average total length	Ratio
			mm.	*mm.*	
Adult male	431	25	2.508	3.230	0.777
Adult female	431	25	2.635	3.315	0.795
" "	358	25	2.720	3.400	0.800
Copepodid V	358	50	2.338	2.933	0.797
" IV	358	13	1.743	2.168	0.805

THE DIURNAL MIGRATION OF COPEPODS IN ST. GEORGES HARBOR, BERMUDA

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The variable nature of the diurnal migration of plankton has rendered difficult the task of discovering the importance of different environmental conditions in causing and controlling the phenomenon. Progress may be made, however, by comparing the behavior of the animals under different circumstances. In view of the data already obtained on the diurnal migration of copepods in the Gulf of Maine (Clarke,1933*b*, 1934), it appeared worthwhile to make similar observations in Bermuda waters, where different species of copepods and entirely different environmental conditions existed. The observations, made in St. Georges Harbor on January 23–24, 1934, must be regarded as approximations since only one (non-closing) net was used. The migration is of such magnitude, however, that there can be no doubt as to its general nature.

The net, kindly loaned to me by Dr. William Beebe, was of the international type. It was 50 cm. in diameter at the mouth and 3 m. long with very coarse netting at the upper end and silk bolting cloth at the middle (12 strands/cm.) and at the bottom (23 strands/cm.). A standard run of a little less than half a mile was laid out between two landmarks in St. Georges Harbor. The tows were made from the Laboratory launch run over this standard course at as nearly the same speed as possible, and always in the same direction, from west to east. Each plankton haul, therefore, was made through the same amount of water. The times required for the run ranged between 10 and 19 minutes, the variability being caused by changing wind conditions. In each set of observations, a haul was made at the surface first and this was followed as quickly as possible by a haul near the bottom. (In one case a third haul was made at a depth halfway to the bottom.) The depth of water was between 12.5 and 14 meters in this part of St. Georges harbor. For the deep haul the net was lowered until the weight, 2 meters below the net, struck the bottom. Then the launch was started and 2 meters more line was payed out. About 20 minutes elapsed between the surface and the deep hauls. The time stated in the table for each set is the midpoint between the times for the two hauls.

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[1] Contribution No. 54.

No attempt was made to study the hydrographic conditions in St. Georges Harbor in detail. The surface temperature was about 20° C. and since a tidal current flowed through the harbor, it is probable that the water was fairly well mixed and hence uniform. A surface haul and a deep haul were taken over the standard run with a diatom net (1 meter long, 30 cm. in diameter, netting 76 strands/cm.). The catches, very kindly analyzed for me by Dr. J. F. G. Wheeler, contained the following total numbers of organisms:

	Surface	Deep
Detritus	Most abundant	Most abundant but less than at surface
Chætoceras (chains)	19,000	27,000
Rhizosolenia	93,000	90,000
Flagellate A. (peridinian?)	79,000	131,000
14 other small forms	24,000	22,000

The analysis of the zoöplankton hauls—carried out by counting and identifying under the microscope either the whole catch or a sample of it—showed the presence of only two species of copepods: *Calanopia americana* (Dahl) and *Acartia spinata* (Esterly). These were divided into adult males and females. Immature specimens were encountered in some of the hauls but were too few to be treated quantitatively. *Calanopia* was found to be the dominant copepod in the bays and harbors of Bermuda at this time (*cf.* Esterly, 1911). It is true that this species was taken in the outside waters but in rapidly diminishing numbers with increasing distance from the mouths of the inland waterways. *Acartia*, on the other hand, was more numerous in the offshore waters and appeared to have been swept into the harbors by the tide.

In the diurnal migration of *Calanopia* and of *Acartia* (Table I) the males and females behaved in the same way. At mid-day the *Calanopia* population could not be found at any depth in the harbor. The animals were undoubtedly so close to the bottom at this time that they could not be caught by the plankton net. In fact, the copepods probably actually burrowed into the soft mud bottom, as is discussed below. At 1944 (local sun time) *Calanopia* was taken in large numbers in the surface hauls and in still larger numbers in the deep hauls. Early the next morning, while it was still dark, the relative distribution of the population was unchanged although all the catches were slightly smaller. Just before sunrise, the numbers of *Calanopia* at both levels were greatly reduced. Although no hauls were taken at noon on the second day, the water was undoubtedly entirely deserted just as before, and at 1707, a quarter of an hour before sunset, the beginnings of the upward migration are apparent. Less than an hour later the numbers of *Calanopia* taken in the surface hauls had increased 10-fold and the numbers taken in the deep hauls had increased 100-fold.

TABLE I

Diurnal migration in St. Georges Harbor, Bermuda. Total numbers of copepods.

	Wind	*Sky*	*Sunrise*	*Sunset*
Jan. 23, 1934..........	SE. moderate	Many clouds	—	1724
Jan. 24, 1934..........	W. slight to strong	Clear	0700	1725

	Local Sun Time	Jan. 23, 1934		Jan. 24, 1934			
		1054	1944	0511	0656	1707	1801
Calanopia americana male	Surface............	0	530	330	15	2	20
	Intermediate.......			2180			
	Deep..............	0	2750	2110	16	17	2340
Calanopia americana female	Surface............	0	740	510	13	3	50
	Intermediate.......			2370			
	Deep..............	1	4380	2370	12	27	2620
Acartia spinata male	Surface............	0	90	50	1	0	0
	Intermediate.......			60			
	Deep..............	30	30	90	120	1	4
Acartia spinata female	Surface............	0	230	60	2	1	2
	Intermediate.......			130			
	Deep..............	40	50	140	180	2	4

The vertical migration of *Acartia* followed the general plan of the *Calanopia* migration but the differences between the day and night hauls are not as striking. At mid-day a considerable number of *Acartia* were taken by the deep net, indicating that this species did not descend to the bottom as completely as did *Calanopia.* At 1944 the upward migration had brought the majority of the animals to the surface. The first set of hauls on the following morning showed a downward movement and this had been greatly accelerated by sunrise. *Acartia* was scarce at 1707 at both levels and even at 1801 no considerable numbers had appeared. This disappearance of the *Acartia* population was coincident with the rising of a strong northwest wind which may have caused a removal of this species, which did not descend entirely to the bottom, through the harbor mouth to the east. On January 23 and on the preceding days, a very strong easterly wind had been blowing. It appears possible, then, that at the beginning of the investigation on diurnal migration there may have been more of this oceanic species present in the harbor than normal, and that during the second day these copepods were being carried back to the outside waters.

Unfortunately it was not possible to make light intensity measurements, but it is clear that the diurnal migration of both *Calanopia* and *Acartia* shows a close correspondence with the rising and setting of the sun. The rapidity of the vertical movements of these copepods is in accord with the shortness of the duration of twilight in low latitudes. The clearness of the water in this part of the ocean (*cf.* Clarke, 1933*a*), coupled with the brilliance of the sun, must have resulted in an intensity of irradiation at all depths in St. Georges Harbor far greater than exists in the surface layers of the Gulf of Maine. It would not be surprising then to find that *Calanopia* had become adapted actually to burrow into the mud during the middle of the day. In fact, during culturing experiments in the laboratory it was found that when individuals of this species were introduced into small glass dishes of sea water with a layer of harbor mud on the bottom, they buried themselves almost immediately. Other species of calanoid copepods did not exhibit this behavior. The animals undoubtedly leave the mud at night for the purpose of feeding (*cf.* Worthington, 1931; Nicholls, 1933). This upward movement might appear superficially similar to the diurnal migrations of certain bottom-living animals (*cf.* Fage, 1933), but Dr. C. B. Wilson informs me that there is every indication that *Calanopia* is a truly pelagic form. As we have seen above, food organisms were present in the water of the harbor, although here as in other warm regions phytoplankton was generally scarce.

To sum up, the copepods *Calanopia americana* and *Acartia spinata* were found to exist in St. Georges Harbor in large numbers near the surface and in the deeper strata at night and to migrate to the bottom at sunrise, the former species apparently burrowing into the mud. As in the Gulf of Maine, the diurnal migration appears to be correlated primarily with changes in light intensity during the course of the day, although it may be partly under the control of other environmental factors as well. The fact that the copepods descend to the bottom is consistent with the great intensity of the submarine irradiation in the Bermuda region.

I am indebted to the Bermuda Biological Station for the use of the facilities of the laboratory and I wish to thank the Director, Dr. J. F. G. Wheeler, for his helpful coöperation during the course of this investigation.

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DIGESTIVE ENZYMES OF THE PELAGIC COPEPOD, CALANUS FINMARCHICUS

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Yonge (1931), in his review of the "Digestive Processes in Marine Invertebrates and Fishes" states that "Little is known of the digestive enzymes of the Entomostraca. . . ." The somewhat incomplete observations here presented are sufficiently illuminating (I believe) to merit publication at this time, particularly since circumstances force me to postpone, for an indefinite period, carrying the work any further.

The feeding mechanism of the calanoid copepods has been described by Storch and Pfisterer (1925), and, perhaps more correctly, by Cannon (1928). They are filter feeders, and their mechanism is so efficient, according to Naumann (1923), that they can remove particles as small as 1 μ in diameter from the water. They can also ingest much larger particles, breaking up and swallowing large diatoms and crustacean nauplii, etc. (Cf. Dakin (1908), Esterly (1916), Lebour (1922), and Marshall (1924) for material observed in the gut.)

Calanus is so small (2.5–6.5 mm.) that it proved impractical to extract the guts alone. Large numbers of the live copepods (copepodid III to adult stages—mostly copepodid IV and V) were separated from other organisms taken in the tows, strained off on bolting silk, and placed in a calibrated test-tube. To each cubic centimeter of copepods was added 0.6 cc. of sea water, 0.4 cc. of 95 per cent alcohol, 1 cc. of toluene, and some glass beads or well washed sand. The tube was stoppered with a rubber stopper (previously boiled in a solution of NaOH and well washed) and shaken violently in a mechanical shaker for an hour or more, until the copepods were reduced to a pink emulsoid. The extraction was then allowed to continue for about 36 hours, and the extract filtered off through filter paper. More toluene was added, and the extract kept at 5–6° C. till used (1 to 7 days). Before use the extract was mixed with an equal volume of buffer. The buffers were made according to the directions given in Clark (1928). Those of pH 1.0, 1.5, and 2.0 were HCl-KCl buffers. For pH 2.4, 2.8, 3.2, 2.6, 4.0, 4.4, 4.8, 5.2, 5.6, and 6.0 the phthalate series was used. For pH 6.4, 6.8, 7.0, 7.2, 7.6, and 8.0 the phosphate series was employed; and for pH 8.49, 8.84, 9.61, 10.96, 11.45, and 12.54 the glycine series.

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[1] Contribution No. 56.

These buffers were used in preference to others because it was known that they have no toxic effect on enzymes.

Since the extract itself was at pH 7.0–7.6, and since it is fairly well buffered, it is probable that the pH of the extract-buffer mixtures in the acid range was actually somewhat higher, and those in the alkaline range somewhat lower than the figures given.

No method was devised for accurately determining the pH of the gut of the living copepods. Neutral red colors the lumen so slightly and the gut is so narrow, that it is impossible, without adequate standards, to be certain that the color observed in the mid-gut was actually (as it appeared) the yellowish red of a somewhat alkaline solution, rather than very dilute brick or rose red of neutral or acid conditions. In the decapods (according to the data summarized by Yonge), for which the stomach pH is known, the normal conditions are acid (5.1–5.6), but it would be unsafe to generalize about other Crustacea from this. It may be said that there is nothing known about gut conditions in general, nor about the enzymes of *Calanus* (as will be seen below) to indicate that the gut pH might not theoretically lie anywhere in a range of from at least as low as 6.0 to at least as high as 8.0.

In the following experiments controls, using buffer alone, and buffer with extract kept 10 minutes in a tube in a boiling water bath, were used except where otherwise noted. The four lots of extract used were checked against each other and found to vary somewhat in strength, but were otherwise similar. Each observation given is based on at least two independent experiments—usually three to six.

Proteinase

The method used was that of Pickford and Dorris (1934). Digestion was found to occur from pH 2.4 to 12.54. Above pH 10.96 there was some alkaline hydrolysis of the substrate, so that it is difficult to assign a value to the action of the extract. Below 2.4 there was acid hydrolysis, but the effect on the gelatin was enough unlike that of the enzyme to make a clear differentiation. The speed of digestion at the various pH values is shown in Fig. 1. It will be noted that there is a peak at pH 3.6–4.0 and a much greater one at 8.0–8.49. The " speed of digestion " as shown in the figure is the reciprocal of the logarithm of the time for complete digestion by the most active lot of extract. As this is only comparative, no numerical scale is given.

Amylase

The method of Pickford and Dorris was used for this enzyme also, cornstarch being the substrate. The complete range of pH values was not tested, but an optimum was found at about 7.2, with a very rapid falling off of activity at 6.0 and a less rapid falling off at 8.0.

Lipase

The ethyl butyrate method previously used by me (1933) was adopted, but without the addition of water. Under the conditions of the experiment, the litmus turned pink only after a few days, but by the end of a week, the difference between the experimental and control tubes was extremely striking.

Alginase

Dr. Selman A. Waksman and his associates have become interested in the polymannuronic anhydride known as alginic acid. This (principally in the form of Ca and Mg salts) may make up 20–30 per cent of the air-dried weight of various marine algæ, such as *Fucus, Sargassum,* etc., and it unquestionably is a very important fraction of the total production of marine plants, corresponding to the stored carbohydrates of terrestrial plants. Of course these larger algæ are not ingested by *Calanus* (except perhaps in the form of fine detritus), but their composition may possibly give some indication of the makeup of the unicellular forms. In any case, it was considered desirable to attempt to discover an alginase in *Calanus.* Dr. Waksman kindly provided me a supply of alginic acid, and also a strain of alginic-acid-digesting bacteria, *Bacterium alginovorum* [Cf. Waksman, Carey, and Allen (1934)].

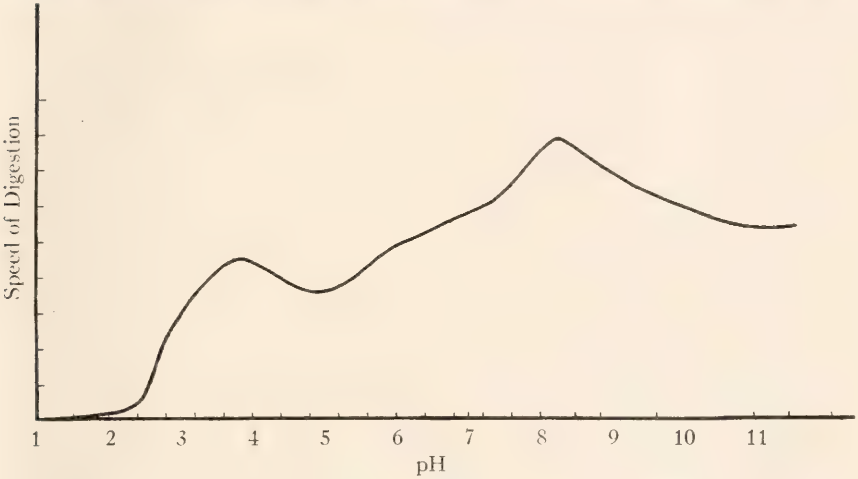

FIG. 1. Graph showing the action of the proteinase of *Calanus* on gelatin.

Agar plate methods were found quite unsuited for the purpose, because neither calcium alginate nor alginic acid remains unchanged by the buffers alone near the neutral point. In dry plates, moreover, the acid becomes practically invisible, and no specific stain is known to me for alginic acid, or its breakdown products.

A set-up was made, however, of 2 cc. of 3 per cent faintly alkaline sodium alginate solution, 1 cc. of buffer at pH 8.0, and 1 cc. of *Calanus* extract. A control with boiled extract was used. Two cc. of the liquid was allowed to drop from a pipette with a finely drawn-out tip, and the meniscus timed between marks with a stop-watch. There was not the slightest detectable change in viscosity during a period of 18 days.

The relative strengths of the enzymes investigated cannot be surely told, because the methods used are not comparable. However, a very dilute extract prepared in a preliminary experiment gave good starch digestion in 20–30 minutes at pH 7.0, whereas no digestion of gelatin occurred at any pH during 16 hours. From this it would certainly appear that the carbohydrate-splitting enzyme is much more vigorous than the proteinase—at least in the latter's action on gelatin.

Digestion of Whole Organisms

Suspensions of living *Nitzschia closterium* (Plymouth strain), *Dunaliella salina*, and *Bacterium alginovorum* were placed in drops of enzyme extract at pH 8.0 in depression culture dishes of soft glass, and the liquid covered by a layer of toluene (which, of course, killed the organisms). The culture dishes were kept at room temperature in Petri dishes containing water and toluene, and the toluene over the extract and in the Petri dishes was renewed from time to time. The controls were in the buffer without extract.

Some of the observations cited above show that *Calanus* guts often contain large numbers of diatoms. *Nitzschia* may be taken as a representative of this group of algæ (though not in itself an important constituent of *Calanus* food). In 36 hours the diatoms in the buffer alone appeared almost normal and stained well—practically like living organisms. In the enzyme, however, the tests appeared empty except for a minute refractive body in the center which took methylene blue extremely lightly—the rest of the diatoms remaining unstained.

The green flagellates after 36 hours were badly broken down both in the enzyme and the control, though those in the enzyme appeared slightly more disintegrated. But, after only 20–24 hours, there was a distinct difference: most of those in the control appearing approximately normal, whereas in the extract, although the chloroplast and other cell inclusions were not noticeably changed, they were crowded more or less into one end of the cell, leaving large clear areas in which no cytoplasm could be made out either without or with methylene blue staining.

The bacteria in the buffer control had settled in 36 hours to the bottom of the drop, and formed a slimy mass, much as they do when alive. In the enzyme the bacteria were perceptibly reduced in numbers (by inspection, to about one-fourth of the control) and were perfectly separate and non-slimy. The individuals remaining, however, took methylene blue very much as the controls did.

Conclusions

Calanus finmarchicus contains enzymes which digest protein, starch, and fat. It has not been shown that these enzymes occur in the lumen of the gut, but that they do is certainly not improbable. Proteolytic digestion is performed by the extracted enzymes from pH 2.4 to above pH 11.0; there is a peak of activity at pH 3.6–4.0, and a still greater peak at pH 8.0–8.49.

Digestion of starch is performed by the extract from slightly below pH 6.0 to above pH 8.0, with an optimum at about 7.2. There is no digestion of alginic acid at pH 8.0, and no evidence was obtained that it is digested at any other pH.

The copepod extract will digest diatoms, bacteria, and at least a part of the cell contents of a green flagellate.

I wish to tender my thanks to the staff of the Woods Hole Oceanographic Institution for the grant of a summer fellowship, and for much invaluable advice and assistance in this work.

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THE DEVELOPMENTAL STAGES OF THE COPEPOD EPILABIDOCERA AMPHITRITES McMURRICH

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(From the University of Washington Oceanographic Laboratories, Seattle, Washington)

INTRODUCTION

Considerable importance is attached to the study of the larval stages of copepods for two reasons: (1) from a taxonomic standpoint the type of larva may throw light on the natural relationship of the adult animals; (2) for the most effective study of the importance of copepods in the economy of the sea it is necessary to take into consideration not only the adult animals but also to distinguish the developmental stages and ascertain the habits and requirements of each.

Epilabidocera amphitrites is a Pacific species occurring on the west coast of North America from whence it was first described by McMurrich (1916) from submature specimens (fifth copepodid) taken off Amphitrite Point, Vancouver Island. The original description was given under the generic name *Paralabidocera,* but Wilson (1932) has pointed out that this name was preoccupied and has therefore proposed the generic name here employed. Adult descriptions are given by Willey (1920) and Esterly (1924).

This species has been reported by Willey (1920) from the northwest coast of Alaska and the Gulf of Alaska, and by Esterly (1924) from San Francisco Bay. As a rule it has not been taken in large numbers but Esterly found it in swarms in San Francisco Bay. Spring swarms also occur at Friday Harbor, Washington.

The writer wishes to thank Dr. T. G. Thompson, Director of the University of Washington Oceanographic Laboratories, for laboratory facilities.

PROCEDURE

In studying the successive nauplius stages, the specimens were taken directly from the plankton at Friday Harbor, and identification of the species was accomplished by selecting specimens of the sixth nauplius stage from fresh plankton hauls and rearing them in section jars through the critical moult to the first copepodid stage. This stage is easily linked to the second copepodid stage, and the following stages are even more readily referable to the adult. Aside from size and pigmentation, the presence of dorsal cuticular lenses and, following the first copepodid stage, the rostral processes and cephalic hooks serve as unmistakable characters identifying the stages with *Epilabidocera amphitrites* which is the only typical species of Pontellidæ that has been found in this immediate region.

All drawings were made with the aid of a camera lucida.

The Nauplius Stages

After the first nauplius stage, the long, slender and straight body with its long caudal spine and the long slender first antennæ make the larvæ of *Epilabidocera amphitrites* conspicuous among the many nauplii occurring in this area. In the living condition, the first antennæ are directed straight forward, and when at rest are in contact practically their whole length. The end segment of these appendages is orange-brown in color and this pigment is also found in the posterior end of the body and to a lesser degree on the ventral side in front of the mouth. The anterior end of the carapace is bent sharply downward forming a curved margin which is slightly constricted on each side of the head. The eye is dark brown and the labrum is long and of medium width. The long bristles occurring on the appendages are plumose but often the fine processes can be discerned only with difficulty. These fine processes have been omitted in the figures except in a few instances where it appeared significant that they be included.

Only a few specimens of Stage I were found and these were all taken from preserved material, hence it is not possible to state with certainty that the pigmentation, etc., described from the living condition for the older specimens applies equally to the first stage.

Stage I (Fig. 1)

Body.—0.182–0.192 mm. (average 0.188 mm.) long, oval with the anterior end slightly the broader, posterior end armed with two straight spines of equal length.

First Antenna.—Three segments, the first short and with one short ventral bristle; the second short and bearing ventrally one very short and one long bristle; the third or distal segment bearing three long terminal bristles and ventrally near the tip a short blunt bristle; dorsally there are two very fine smooth bristles and on the inner margin posterior to the middle there is situated a transverse series of very minute spines.

Second Antenna.—First basipod with one masticatory hook. Second basipod with one masticatory hook, an adjacent small outwardly directed spine and one small spine near the distal end. Endopod of one segment with two long terminal bristles and one long lateral bristle.

Exopod of six segments, the first short with no bristle, 2–5 with one bristle each, and the sixth with two bristles.

Mandible.—First basipod with a small chewing process bearing a small spine. Second basipod with two spines on the inner side. Endopod of two completely fused segments, the first with two weak spines and the second with one short and two long bristles. Exopod of four segments, 1–3 with one bristle each, the fourth with two bristles.

Stage II (Fig. 2)

Body.—0.253–0.274 mm. (average 0.264 mm.) in length, slender, evenly tapered posteriorly and terminating in one long heavy setose spine and, to the right of it, one shorter dorsally directed plumose bristle. At the base of each of these there are a number of very short spines. Two series of similar short spines also appear on each side of the body in the posterior lateral region (Fig. 2, *ls*).

First Antenna.—The first and second segments as in Stage I; the distal segment bears at the tip three long bristles and one shorter accessory bristle, and near the tip there are two fine bristles on the dorsal and two on the ventral margin. Each margin also bears a separate row of fine hair-like bristles. The inner transverse series of minute spines as in I.

Second Antenna.—First basipod with one masticatory hook and a small outwardly directed spine near the base of the hook. Second basipod as in I. Endopod with two long terminal bristles, and on the lateral margin, the one long bristle of Stage I is replaced by one short and two long hair-like bristles and an adjacent series of minute bristles. Exopod as in I but with two bristles on the second segment.

Mandible.—First and second basipods as in I. Endopod as in I but with three strong hooked spines on the first segment and two long

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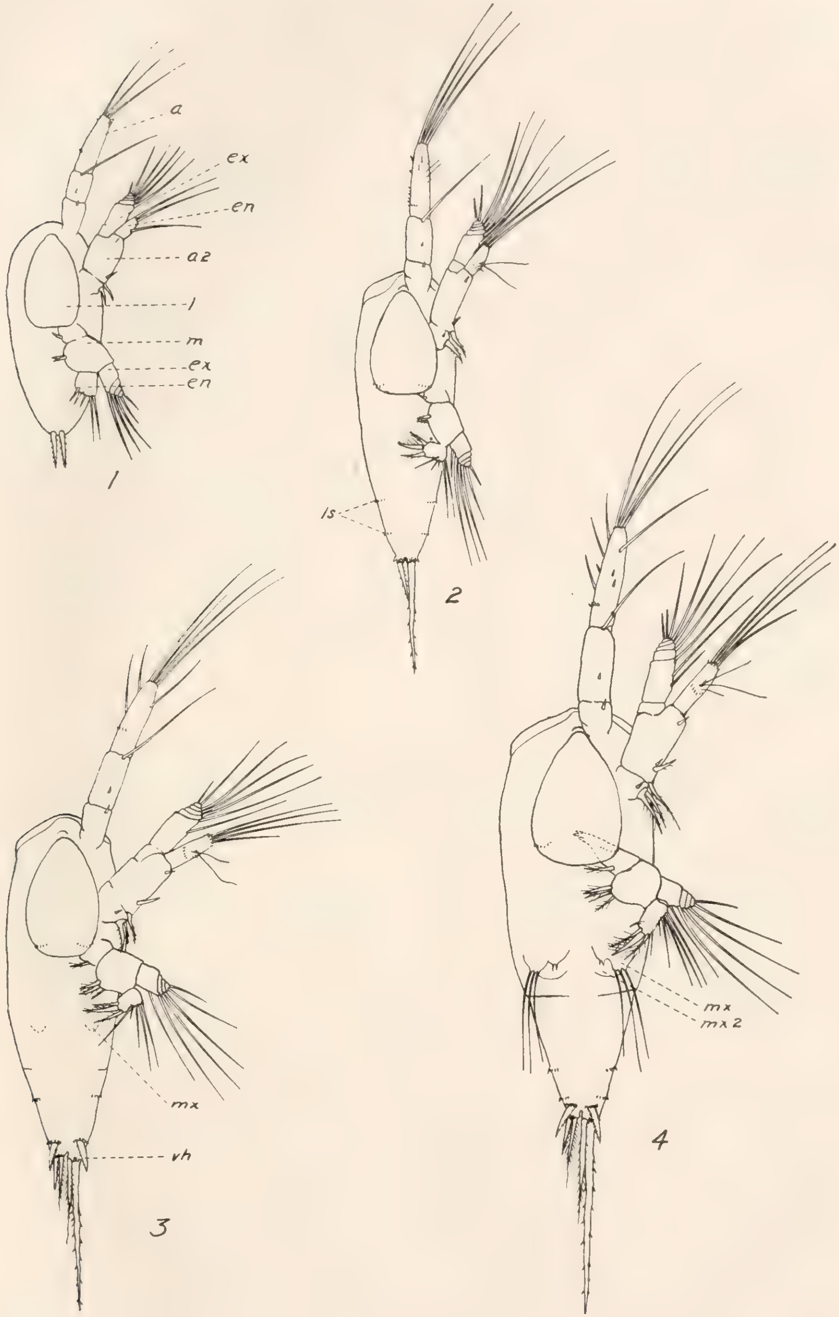
PLATE I

Epilabidocera amphitrites

FIG. 1. Nauplius Stage I.
FIG. 2. Nauplius Stage II.
FIG. 3. Nauplius Stage III.
FIG. 4. Nauplius Stage IV.

Abbreviations:
a—first antenna.
a2—second antenna.
en—endopod.
ex—exopod.
l—labrum.
ls—series lateral spines.
m—mandible.
mx—first maxilla.
mx2—second maxilla.
vh—ventral hooks.


a
ex
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4

PLATE I

and three shorter bristles on the second segment. Exopod as in I but with two bristles on the first segment.

Stage III (Fig. 3)

Body.—0.312–0.361 mm. (average 0.337 mm.) long, posterior end armed with one long and one short terminal setose spine and between these two plumose bristles, and just anterior to them one pair of ventral hooks. Only one series of minute spines occurs on each side of the body in the posterior lateral region.

First Antenna.—Segments one and two as in II; the distal segment with four terminal bristles as in II, two long dorsal marginal bristles, one long ventral marginal bristle and an inner transverse series of minute spines. The fine hair-like bristles found on the distal segment in stage II are wanting.

Second Antenna.—First basipod with one strong masticatory hook, a short hook placed upon its base and a small outwardly directed spine near its base. Second basipod as in II. Endopod as in II but with one medium and three long terminal bristles. Exopod as in II.

Mandible.—First basipod with the chewing process somewhat enlarged and bearing a spine. Second basipod with three inner spines. Endopod and exopod as in II.

First Maxilla.—Bud (?).

Stage IV (Fig. 4)

Body.—0.380–0.412 mm. (average 0.402 mm.) long, posterior end armed as in III but with two series of minute spines on each side of the body in the posterior lateral region.

First Antenna.—The first and second segments as in III; the distal segment with four terminal bristles, one short and three long dorsal marginal bristles and a transverse series of minute spines on the inner side.

Second Antenna.—First basipod as in III. Second basipod as in III but with an additional short spine. Endopod as in III. Exopod as in III but with three bristles on the second segment.

Mandible.—First basipod with a long, strong chewing process or blade toothed at the end and bearing a spine near the base. Second basipod with one weak and three stronger spines. Endopod with three strong hooked spines and two weak bristles on the first segment and four long and two short very weak bristles on the second segment. Exopod as in III.

First Maxilla. Rudimentary with two lobes, the outer lobe with three long weak bristles and the inner lobe with two very short weak bristles.

Second Maxilla.—Bud.

Stage V (Fig. 5)

Body.—0.421–0.500 mm. (average 0.481 mm.) long, posterior end armed as in IV but with the addition of a pair of lateral hooks similar to the ventral pair. Just anterior to the lateral hooks there remains but one series of minute spines on each side of the body.

First Antenna.—Segments 1 and 2 as in IV; the distal segment with four terminal bristles, four long and two short dorsal marginal bristles, one long and two short ventral marginal bristles and a transverse series of minute spines on the inner side.

Second Antenna.—As in IV but with five terminal bristles on the endopod.

Mandible.—As in IV but with the addition of one very weak bristle on the second basipod.

First Maxilla.—Rudimentary, the outer and inner lobe with five and two long bristles respectively.

Second Maxilla.—Bud.

Maxilliped.—Bud.

Stage VI (Fig. 6)

Body.—0.540–0.575 mm. (average 0.564 mm.) long, posterior end armed as in V.

First Antenna.—The first and second segments as in V; the distal segment with four terminal bristles, five long and three short (alternating with the long) dorsal marginal bristles, one long and four short ventral marginal bristles and a transverse series of minute bristles on the inner side.

Second Antenna.—As in V but the endopod with four hair-like bristles and a group of very fine short bristles on the lateral margin.

Mandible.—As in V.

First Maxilla.—Rudimentary, the outer and inner lobe with seven and four bristles respectively.

Second Maxilla.—Bud.

Maxilliped.—Bud.

First and Second Feet.—Rudimentary.

The Copepodid Stages

The sixth nauplius stage terminates in the critical moult from which the first copepodid emerges in the form of the adult but lacking in certain characters relating to the number of appendages, segmentation, etc. There is a total of six copepodid stages, the last of which is the adult animal. Throughout these stages the animal remains relatively slender and tapered at the anterior end. In the living condition the urosome and caudal rami are orange-brown in color, the pigment usually extending into the caudal bristles. It is also present in the distal segment of the first antenna in the younger stages (and in the right antenna of the adult male) and in the anterior part of the head, extending in increasing intensity from behind the cuticular lenses towards the rostrum.

Stage I (Figs. 7 and 8)

Body Length.—To end of caudal rami 0.76–0.82 mm., thorax[1] of four segments, abdomen of one segment, dorsal cuticular lenses present but the rostral processes and lateral cephalic hooks which are typical of the later stages are wanting. The anterior end of the head is flexed downward forming a blunt rounded knob-like rostrum. The urosome and caudal rami are symmetrical.

First Antenna.—Ten segments.

Second Antenna.—First basipod with one bristle. Second basipod with two bristles. Endopod of two segments, the first with two bristles; the second forming two lobes, the inner lobe with three long and one shorter bristle, the outer lobe with five long and one shorter bristle. Exopod of five segments, the first short and bearing one bristle, the second long and with two lateral and two distal bristles, the third and fourth with two bristles each and the fifth with two terminal bristles.

Mandible.—Mandibular blade with five teeth; palp with a short first basipod, long second basipod and two short rami, the inner ramus with a terminal group of four bristles and a lateral group of four bristles, the outer ramus with six bristles.

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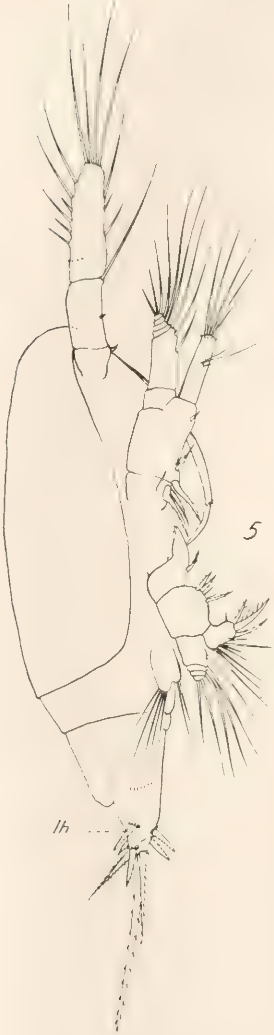
[1] The thorax of the adult copepod is considered to consist of six segments.

In my previous paper (Johnson, 1934) the term 'urosome' is applied to that portion of the body posterior to the last thoracic segment bearing visible feet. The term 'thorax' used in explanation of Figs. 2–6, Plate III, should read 'metasome.'

PLATE II

FIG. 5. Nauplius Stage V.
FIG. 6. Nauplius Stage VI.
FIG. 7. Copepodid Stage I, lateral.
FIG. 8. Copepodid Stage I, dorsal.

Abbreviations:
f—first feet.
f2—second feet.
lh—lateral hooks.
mp—maxilliped.
mx—first maxilla.
mx2—second maxilla.



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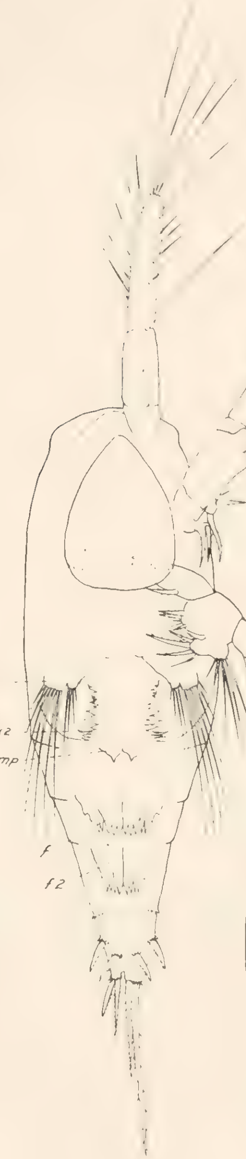
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mx2
mp
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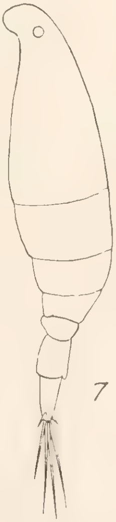
f2

6



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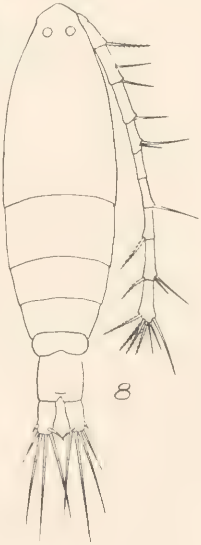

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PLATE II

First Maxilla.—All lobes formed as in the adult but each with fewer bristles except the inner lobe of segment two and segment three, each of which bears three bristles as in the adult.

Second Maxilla.—As in the adult with fifteen long, strong bristles.

Maxilliped (Fig. 10).—Uniramose with a total of four segments; the first lobe of the first basal with one short setose bristle, the second lobe of the first basal with one short and one long setose bristle, the third lobe of the first basal with one long setose bristle and a very small bristle at its base; the second basal with one distal plumose bristle; the third segment with one distal plumose bristle; the distal segment with three terminal smooth bristles.

First Feet.—First and second basipods with no bristles. Endopod of one segment with six bristles. Exopod of one segment with four outer spines, a terminal blade, and three bristles.

Second Feet.—First and second basipods with no bristles. Endopod of one segment with six bristles. Exopod of one segment with three outer spines, a terminal blade, and three bristles.

Third Feet.—Rudimentary.

Stage II

Body.—1.01–1.16 mm. long, thorax of five segments, abdomen of one segment; dorsal cuticular lenses, rostral processes and lateral cephalic hooks present (Fig. 9). Urosome and caudal rami symmetrical.

First Antenna.—Fourteen segments.

Second Antenna.—As in I but with three long and two shorter bristles on the inner lobe of the distal segment of the endopod.

Mandible.—As in I but the inner branch of the palp with five long and two short bristles in the terminal group.

Maxilliped.—A total of five segments; the first basal as in I but with two short setose bristles on the first lobe; the second basal with a serrated margin and one distal plumose bristle; the third and fourth segments each with one distal plumose bristle and the fifth with three smooth terminal bristles.

First Feet.—First basipod with one inner bristle. Second basipod with no bristle. Endopod of two segments, the first with one inner bristle, the second with seven bristles. Exopod of two segments, the first with one outer thorn, the second with three outer thorns, a terminal blade and four bristles.

Second Feet.—First basipod with one inner bristle. Second basipod with no bristle. Endopod of one segment with seven bristles. Exopod of two segments, the first with one outer thorn, the second with two outer thorns, a terminal blade and four bristles.

Third Feet.—First and second basipods with no bristles. Endopod of one segment with six bristles. Exopod of one segment with three outer thorns, a terminal blade, and three bristles.

Fourth Feet.—Rudimentary.

Stage III

Body.—1.43–1.50 mm. long, thorax of six segments, abdomen of one segment. Cuticular lenses, rostral processes and cephalic hooks present. Posterior end of body symmetrical.

First Antenna.—Sixteen segments.

Second Antenna.—As in II.

Mandible.—As in II.

Maxilliped.—A total of six segments, otherwise as in II.

First Feet.—First basipod with one inner bristle. Second basipod with no bristle. Endopod of two segments, the first with one inner bristle, the second with eight bristles. Exopod of two segments, the first with one outer thorn and one inner bristle, the second with three outer thorns, a terminal blade and four bristles.

Second Feet.—First basipod with one inner bristle. Second basipod with no bristle. Endopod of one segment with nine bristles. Exopod of two segments, the first with one outer thorn and one inner bristle, the second with three outer thorns, a terminal blade and five bristles.

Third Feet.—First basipod with one inner bristle. Second basipod with no bristle. Endopod of one segment with seven bristles. Exopod of two segments, the first with one outer thorn, the second with two outer thorns, a terminal blade and four bristles.

Fourth Feet.—First and second basipods with no bristles. Endopod of one segment with six bristles. Exopod of one segment with three outer thorns, a terminal blade, and three bristles.

Fifth Feet.—Rudimentary.

Stage IV

Body.—2.23–2.35 mm. long, thorax of six segments (the fifth thoracic segment symmetrical with slightly pointed corners), abdomen of two segments in both sexes.

First Antenna.—Twenty-three to twenty-four segments.

Second Antenna.—As in III but with an additional bristle in each of the two lobes of the terminal segment of the endopod.

Mandible.—As in III.

Maxilliped (Fig. 11).—As in III but with one very small bristle at the base of the long bristle on the second lobe of the first basal and two very small bristles at the base of the long bristle on lobe three; basal two has three distal plumose bristles and segment three has two distal plumose bristles.

First Feet.—First basipod with one inner bristle. Second basipod with no bristle. Endopod of two segments, the first with one inner bristle, the second with eight bristles. Exopod of two segments, the first with one outer thorn and one inner bristle, the second with three outer thorns, a terminal blade and four bristles.

Second Feet.—First basipod with one inner bristle. Second basipod with no bristle. Endopod of one segment with ten bristles. Exopod of two segments, the first with one outer thorn and one inner bristle, the second with three outer thorns, a terminal blade, and five bristles.

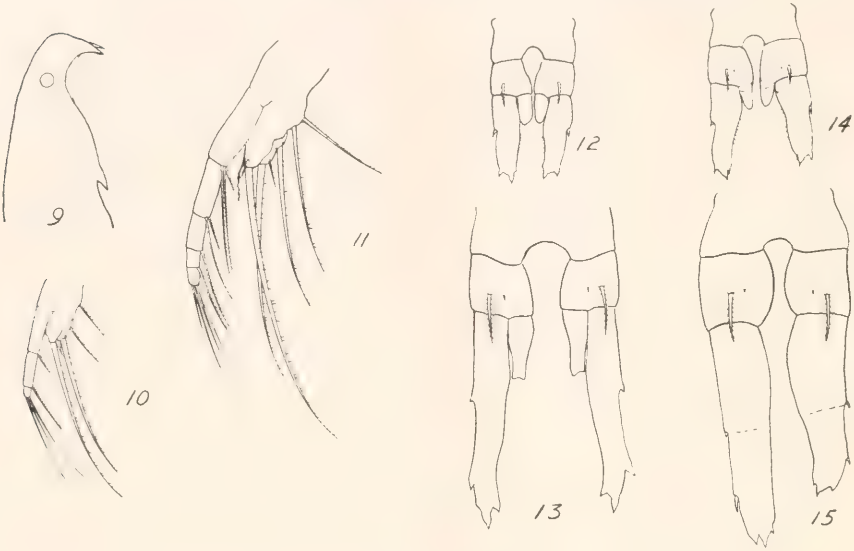

PLATE III

Fig. 9. Head copepodid Stages II–VI, lateral.
Fig. 10. Maxilliped copepodid Stage I.
Fig. 11. Maxilliped copepodid Stage IV.
Fig. 12. Fifth feet copepodid Stage IV, female.
Fig. 13. Fifth feet copepodid Stage V, female.
Fig. 14. Fifth feet copepodid Stage IV, male.
Fig. 15. Fifth feet copepodid Stage V, male.

Third Feet.—First basipod with one inner bristle. Second basipod with no bristle. Endopod of one segment with nine bristles. Exopod of two segments, the first with one outer thorn and one inner bristle, the second with three outer thorns, a terminal blade and five bristles.

Fourth Feet.—First basipod with one inner bristle. Second basipod with no bristle. Endopod of one segment with eight bristles. Exopod of two segments, the first with one outer thorn, the second with three outer thorns, a terminal blade and five bristles.

Fifth Feet, Female (Fig. 12).—Symmetrical, biramose, the inner ramus being short and rounded at the tip, the outer ramus of one segment with one outer thorn and one small and two large spines at the tip, and on the inner margin a number of fine hairs. The posterior surface of the second basipod bears one long and one very short bristle.

Fifth Feet, Male (Fig. 14).—Symmetrical, biramose, the inner ramus short and rounded as in the female, the outer ramus as in female but with two large and two very small spines at the tip; second basipod with bristles as in the female.

Stage V

Body.—2.73–3.30 mm. long, thorax of six segments, the fifth thoracic segment asymmetrical in the male due to increased length of the right corner forming a conspicuous point; abdomen symmetrical and of two segments in the female and three in the male.

First Antenna.—Twenty-four segments.

Second Antenna.—As in IV but with seven and eight bristles respectively in the inner and outer lobes of the distal segment of the endopod.

Mandible.—As in IV.

Maxilliped.—As in IV.

First Feet.—First basipod with one inner bristle. Second basipod with no bristle. Endopod of three segments, the first with one inner bristle, the second with two inner bristles, the third with six bristles. Exopod of three segments, the first and second each with one outer thorn and one inner bristle, the third with two outer thorns, a terminal blade and five bristles.

Second Feet.—First basipod with one inner bristle. Second basipod with no bristle. Endopod of two segments, the first with three inner bristles, the second with eight bristles. Exopod of three segments, the first and second each with one outer thorn and one inner bristle, the third with three outer thorns, a terminal blade and five bristles.

Third Feet.—First basipod with one inner bristle. Second basipod with no bristle. Endopod of two segments, the first with three inner bristles, the second with eight bristles. Exopod of three segments, the first and second each with one outer thorn and one inner bristle, the third with three outer thorns, a terminal blade and five bristles.

Fourth Feet.—First basipod with one inner bristle. Second basipod with no bristle. Endopod of two segments, the first with three inner bristles, the second with seven bristles. Exopod of three segments, the first and second each with one outer thorn and one inner bristle, the third with three outer thorns, a terminal blade and five bristles.

Fifth Feet, Female (Fig. 13).—Symmetrical, biramose, the inner ramus short and with two short points, the outer ramus of one long segment with one outer spine near its middle and one inner and one outer spine near the one terminal spine; second basipod with bristles as in IV.

Fifth Feet, Male (Fig. 15).—Asymmetrical, uniramose, each foot consisting of first and second basipods with bristles as in the female, and two firmly fused segments of the exopod. Distally the first segment has an outer spine, the second segment bears one outer lateral spine, two terminal spines, and one very small inner spine near the tip.

Stage VI, Adult

Discussion

Epilabidocera amphitrites possesses a typical calanoid nauplius larva. The body shape resembles most closely the larvæ of *Pontella mediterranea, Centropages hamatus, Acartia longiremis* and *Tortanus discaudatus* in having a straight hind body which is not sharply marked off from the fore body as occurs in *Calanus finmarchicus* and *Temora longicornis*. A comparison with the incomplete figures of Claus (1893, Plate I) indicates a striking similarity to the late nauplius of *P. mediterranea*. In both species the body is unusually slender and the caudal armature is apparently of the same type. The caudal armature also recalls that of *C. hamatus* and *T. longicornis* in the presence of a long heavy left terminal spine. In Stage II this likeness to the corresponding stage is greater in the former species, whereas the following stages recall more nearly the larva of the latter. In the latter species, however, the short right spine partakes less of the character of a " hook " and there is a maximum of three pairs of lateral hooks and two pairs of ventral hooks as contrasted with one pair each in *E. amphitrites*. Another similarity is seen in the sixth stage when both species bear eight dorsal marginal bristles and five ventral marginal bristles on the distal segment of the first antenna, but *E. amphitrites* is unique in having an alternation of long and short bristles on the dorsal margin.

The likeness of the caudal armature to that of *C. hamatus* may be said to diminish after the second stage, and yet this dissimilarity is not so great if the end spines (" Endborsten ") which appear in the third stage of *C. hamatus* (Oberg, 1906, Plate II, Fig. 3) can be considered distally placed lateral hooks, as they appear to be.

Table I gives a brief summary of the characters useful in identification of the successive nauplius stages of *E. amphitrites*.

TABLE I

Identification Table for Nauplius Stages of Epilabidocera Amphitrites

Stage No.	I	II	III	IV	V	VI
Length in mm.	0.182–0.192	0.253–0.274	0.312–0.361	0.380–0.412	0.421–0.500	0.540–0.575
First antenna distal segment	3 terminal bristles	4 terminal bristles, 1 dorsal and 1 ventral series of fine hairs	4 terminal bristles, fine hairs wanting, 1 ventral and 2 dorsal marginal bristles	As in III, but 4 dorsal and 3 ventral marginal bristles	As in IV, but 6 dorsal and 3 ventral marginal bristles	As in V, but 8 dorsal and 5 ventral marginal bristles
Caudal armature	2 equal end spines	1 long end spine, 1 shorter end bristle, 2 pairs series minute lateral spines	1 long and 1 short end spine, 2 end bristles, 1 pair ventral hooks, 1 pair series minute lateral spines	As in III, but with 2 pairs series minute lateral spines	As in IV, but also 1 pair lateral hooks and only 1 pair series minute lateral spines	As in V
First maxilla	0	0	Bud?	Rudimentary	Rudimentary	Rudimentary
Second maxilla	0	0	0	Bud	Bud	Rudimentary
Maxilliped	0	0	0	0	Bud	Bud
First and second feet	0	0	0	0	0	Rudimentary

The copepodid stages follow the regular order of development. In the development of the feet, it will be noted that upon first appearance (after the rudimentary stage) each ramus of each pair of feet possesses but one segment. With the next moult the exopod (and also the endopod in the first pair) divides once and remains thus through the subsequent moults to the fifth stage, when each branch of all the feet (excepting the fifth pair) add one more segment, giving the total number found in the adult. This was also the method of development of the first four pairs of feet in *Tortanus discaudatus* (Johnson, 1934). In *Calanus finmarchicus* the development is similar to this except that the moult immediately following the appearance of the feet gives rise to two segments in both rami of each pair of feet. After this they remain unchanged until the fifth stage when the adult condition is acquired by the addition of one more segment to each branch (Lebour, 1916, Plate V). *Centropages* evidently also follows this routine (Oberg, 1906, Plate VII, Fig. 7, *A–F*).

In the sixth stage of *E. amphitrites* the fifth feet of the male undergo drastic modification upon reaching maturity. It is of interest to note that in the fourth copepodid stage the fifth feet are practically identical in the sexes, both possessing a short inner ramus. There is a slight difference relative to the terminal spines of the exopod. In this stage the sexes can be distinguished best by the slightly swollen right antenna of the male. In the fifth stage the male has completely lost the inner ramus, whereas in the female it has developed somewhat but still remains rudimentary. The appearance and subsequent disappearance of the inner ramus in the male suggests a stronger tendency in the female to retain the primitive character in this respect.

Up to the adult stage the urosome is symmetrical in both sexes. The only obvious asymmetry in the body in the fifth stage is found in the fifth thoracic segment of the male, which is drawn out to a longer point on the posterior margin of the right side. In the adult the urosome is very asymmetrical, particularly in the female.

The amount of asymmetry found in the nauplius stages of some copepods may not be especially significant in indicating the extent of asymmetry present in the adult. Adult asymmetry comes mainly in the sixth or adult stage and appears to be closely associated with sexual maturity. It must be said, however, that where marked asymmetry occurs in the nauplius stages, some type of body asymmetry can be expected in the adult animal or in one or more species within the family or genus to which it belongs, but not necessarily to the same species. For example, the nauplius stages of *Temora longicornis, Centropages hamatus*, and *Epilabidocera amphitrites* are strikingly asymmetrical, and

Table II

Identification Table for Copepodid Stages of E. amphitrites

Stage No.	I	II	III	IV	V	VI
Length in mm.	0.76–0.82	1.01–1.016	1.34–1.50	2.23–2.35	2.53–3.30	3.2–4.0
First antenna	10 segments	14 segments	16 segments	23–24 seg. right in male slightly swollen	24 seg. right in male swollen	24 seg. right in male geniculated
Feet present	1st and 2d (3d rudimentary)	1st, 2d and 3d (4th rudimentary)	1st, 2d, 3d and 4th (5th rudimentary)	1st, 2d, 3d, 4th and 5th	As in IV but differ in sexes	As in V
Thorax	4 segments	5 segments	6 segments	6 segments	6 segments	6 segments
Abdomen	1 segment	1 segment	1 segment	2 segments	2 seg. ♀ 3 seg. ♂	Asymmetrical 2 segments 4 segments

of the adults *T. longicornis* is usually symmetrical (but in *T. turbinata* the anal segment is somewhat asymmetrical in the female); *C. hamatus* is slightly asymmetrical in the genital segment and in the posterior corners of the last thoracic segment of the female; and *E. amphitrites* is very asymmetrical. *Tortanus discaudatus* and *Acartia longiremis* possess relatively little asymmetry in the larvæ, while the urosome and caudal rami of the former are very distorted in both sexes in the adult, and only a little tendency to distortion is seen in the caudal rami of the female in the latter.

Calanus finmarchicus and *Pseudocalanus minutus* (syn. *elongatus*) represent the calanoid group with symmetrical bodies when mature and little or no asymmetry in the nauplius stages (Oberg, 1906; Lebour, 1916).

The typical harpacticid nauplius (Gurney, 1930, *Longipedia,* Fig. 10) and adult are symmetrical, and the same is true of the Cyclopoida (Gibbons and Ogilvie, 1933, *Oithona helgolandica* and *O. spinirostris,* Plates I and II).

From the above few comparisons it is seen that, in so far as the life histories of marine copepods have been investigated, there appears to be some tendency to larval asymmetry in the calanoid group in which some adults are asymmetrical. Whether or not there is a period in the early copepodid stages in which the asymmetry remains latent, only additional investigation on life histories will show. It must be borne in mind that the asymmetrical calanoids (for example, the Pontellidæ) usually also possess striking modification in secondary sex characteristics relative to the feet and antenna which are not indicated in the larvæ, and that the body asymmetry may develop in the adult due to the same more recently acquired trend.

Table II gives briefly the most useful characteristics for identification of the copepodid stages of *Epilabidocera amphitrites.*

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PULSATIONS IN THE NEREIS EGG

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It is a well-known fact that the eggs of many forms change their shape markedly in the period between the time of fertilization and first cleavage. This is more noticeable in the eggs of some animals than in those of others. It is especially evident in such an egg as that of *Chætopterus,* where a polar lobe is formed just prior to the first cleavage. In the eggs of annelids and mollusks it is quite usual for the formation of the polocytes to be accompanied by changes in the gross shape of the egg, which becomes somewhat flattened just before the appearance of the polar elevation.

Movements of the cytoplasm of the central and more peripheral regions have been described in a large number of eggs. Many of these are mass movements which result in the isolation of specific regions. This is well illustrated by the processes which lead to the formation of the yellow crescent of the egg of *Styela* and of the grey crescent of eggs of certain amphibia. Similarly, mass movements and migration of regions have been noted in the developing egg of *Chætopterus* (Lillie, 1906) and the ctenophore (Spek, 1926). Such mass movements are slightly different from those characteristic of the telolecithal eggs of certain fishes and the squid, where the first visible evidence of activation and development comes with the streaming of the cytoplasm and its subsequent accumulation in the blastodisc.

Changes in the gross shape of the egg or of parts of the developing germ may or may not be related to the isolation of specific regions. In the coelenterates, constant movement is not uncommon, the most extensive being found in *Hydra,* which is "amoeboid" during its early development. The deformation of the egg during polar body elevation and that accompanying the formation of the polar lobe are essentially the same, though the former is not immediately related to any developmental restriction. There is still another group of movements involving altered relationships between parts of the cytoplasm. They appear at the time of cell division and are definitely related to cleavage. Such flowing movements are probably of general occurrence and have been described and discussed by so many workers that no specific examples need be cited here.

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[1] This investigation was aided by grant from the Milton Fund.

The present paper is a report of changes in the gross shape of the egg of *Nereis* which take place in the interval between sperm attachment and the second cleavage. Though this activity is not apparent on casual observation, it proves to be very extensive when camera lucida drawings of the egg are made at short intervals and compared. The search for indications of activity was prompted by the observation of certain phenomena associated with the division of the cell in the Canti Tissue Culture Film and with the development of the egg of the rabbit as seen in the film by Professor Warren Lewis and Dr. Paul W. Gregory.

The egg of *Nereis* was selected for use in making these observations for two reasons. This egg is inseminated while the germinal vesicle is intact, and hence the period of observation includes not only the time of first and second cleavage but also of maturation. As will be seen below, these periods are of great interest. Moreover, the egg of *Nereis* is enclosed within an easily determined membrane and contains many oil droplets which are constant in their position. Both supply reference points on which one may depend in determining, first, the extent of the deformation of the egg and, second, its constant orientation in the field of the microscope.

The eggs were prepared for observation in the following way. A large female collected the previous night was placed in a finger bowl with an active male. Both were immediately excited and shedding commenced. A stop watch was used for timing observations. Approximately a minute after the first appearance of the eggs, a very few were placed on a slide in a considerable amount of sea water. The cover glass was supported and sealed on three sides with vaseline. Eggs treated in this way complete the first cleavage at the same time as the controls. The first batch of eggs tested was placed on a slide without the precaution of the vaseline seal. Very interesting information was obtained from this and similar experiments which will be considered below.

Inasmuch as but a few of the eggs were placed under the cover glass, not more than two or three at most were included in one field of the microscope and then, often only by the use of the low power. The manipulation and selection of the eggs to be observed required some five minutes so that observations usually began at about that time. While watching the eggs, it is relatively simple to tell in just what regions changes are taking place, for, aside from the oil droplets, the edge of the fertilization membrane gives a reference line to which changes in shape may be referred. Both oil droplets and membrane were used in continuous observation but their use in making records had to be abandoned. It was soon found that the time required to draw two sets of lines was too great to be practical. Speed was required and hence eggs were drawn in the same orientation without reference lines. Unfortunately, alterations in contour which appear great on observation of the egg within the membrane are not as evident when the outline of the egg alone is examined. In order to minimize any error which might arise from haste and slight inaccuracies in tracing, an initial magnification of 200 diameters was employed and the sketches made by means of a camera lucida. Subsequent photographic reduction has led to more accuracy than could have been hoped for at the magnification given in the text figure. An effort was made to draw the egg at 20 to 30-second intervals up to the time of first cleavage. The number given in each of the accompanying sketches represents the time in minutes and seconds which elapsed between the initiation of shedding by the female and the completion of the sketch. Complete pictorial histories of individual eggs, while a part of the records, are not included here for obvious reasons.

In making observations of this kind it is well to bear in mind several possible sources of error. If the orientation of the egg changes during the course of the observation, no positive information is obtained inasmuch as the turning of the egg may lead to changes in shape which are apparent rather than real. In like manner it is wise to be very cautious in recording swelling and shrinking of the eggs, for these may be the result of modifications in the shape which would be evident if viewed from the side, but which may readily be misinterpreted when seen from above. Both of these effects have been controlled in the present observations.

Observations

The observations described below were made at the Marine Biological Laboratory during two successive summers. The results are similar and so consistent that part of but one series of observations need be considered in any detail. A general statement of the findings will be presented first.

The development of the egg of *Nereis* is so well known that but an outline need be presented here. If the cultures are maintained at a temperature of from 18° to 19° C., the main events which serve for the identification of the various stages occur approximately as seen in Table I. All of the events recorded in the table are separated by appreciable intervals of time save for the final engulfment of the sperm head and first polar body formation. For this reason the 100-minute period has been subdivided for convenience into three shorter intervals indicated in the table as 'A,' 'B,' and 'C.' The first ('A') covers the time elapsing between the insemination of the eggs and the final incorporation of the sperm head within the cortex of the egg. This includes the breakdown of the germinal vesicle. The second period ('B') begins with the isolation of the first polar body as a discrete unit and ends with the elevation of the second polar protuberance. In reality the period stops just before the elevation of the cytoplasm but the egg must be followed to that stage before the exact degree of development can be determined. The third interval ('C') covers the period from the complete isolation of the second polar body up to the time of the deformation of the egg prior to the first cleavage. We shall now consider in a general way the events in each of the three intervals.

TABLE I

Interval	Event	Time after insemination
		minutes
	Insemination of the egg	0
A	Breakdown of the germinal vesicle	15–20
	Final incorporation of sperm	40–45
B	First polar body formation	45–50
C	Second polar body formation	60–65
	First cleavage	90–100

The interval designated above as 'A' is characterized by certain changes in the nucleus of the egg and by the gradual incorporation of the sperm. The germinal vesicle breaks down as indicated in the table and the contents of this cell organ approach the surface of the egg near the polar end. Toward the latter part of the interval the spindle of the first polocytic division forms from this material. In the meantime the cytoplasm of the egg itself displays an activity which appears to be just as characteristic. Immediately before and during the breakdown of the germinal vesicle, the egg goes through a series of slow labored pulsations which deform the egg, now in one direction, now in another. Some of these are quite extensive. The deformations appear at their greatest just before and during the actual disappearance of the membrane of the vesicle. They continue up to approximately 25 minutes after insemination, when the egg appears to enter a short 'rest period' during which they practically cease. The egg emerges from the 'rest period' after from 3 to 5 minutes and upon regaining the active state continues changing its shape by pulsating without pause throughout the remainder of the period which ends at about the time of the disappearance of the sperm head within the egg. At no time are the movements as great following the 'rest period' as during the breakdown of the vesicle. There is some difference between the general appearance of the pulsations which appear prior to the 'rest period' and after. These will be considered below.

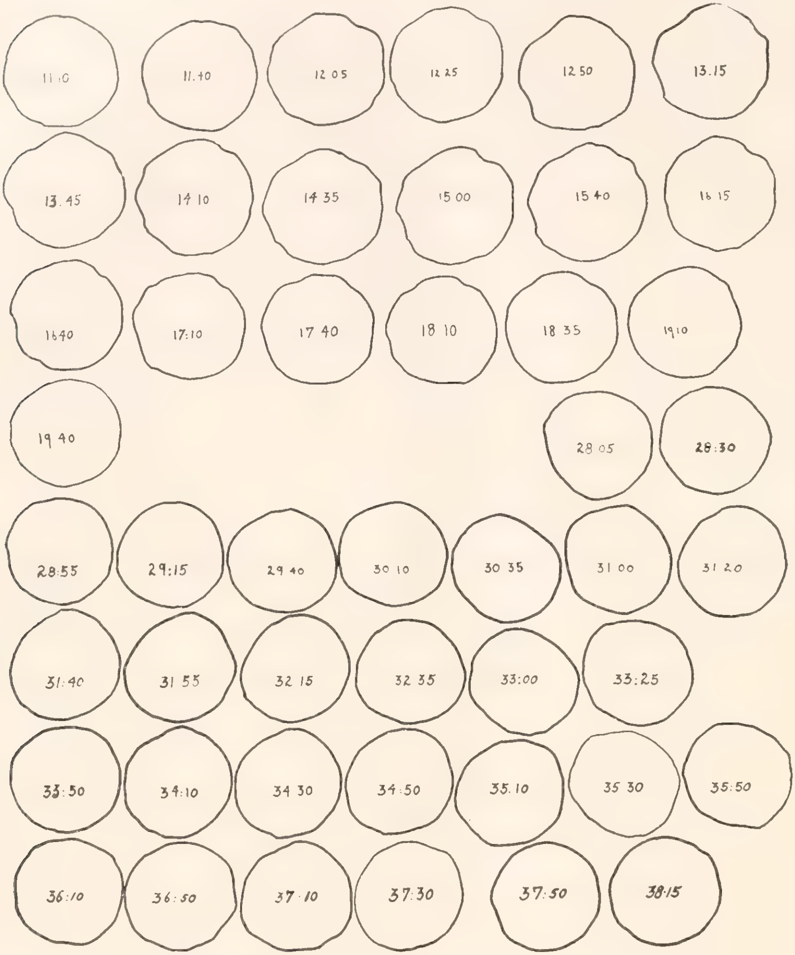
The second interval (interval 'B') covers the period between the complete separation of the first polar body and the initiation of the elevation of the second. During this period, as during the first, the activities of the zygote may be seen to center around the female nucleus, the sperm head, and the cytoplasm of the egg. The egg nucleus, now that of the secondary oöcyte, forms the second maturation spindle. The sperm head swells and becomes reoriented within the outer layers of the cytoplasm near the site of penetration. In addition, the interval is characterized by a succession of pulsations of the same sort as those noted during the first interval. The pulsations are probably even less extensive than those following the 'rest period' of interval 'A' though the difference is slight. There is no doubt, however, but that the interval represents a period of increased activity so far as egg movement is concerned.

Interval 'C' extends from the completion of second polar body formation to the beginning of the first cleavage. It is characterized within the nuclear elements of the zygote by the formation of the female pronucleus, the establishment of the male pronucleus, the migration of both to a central position in the egg with the development of the asters, and the breakdown of the united nuclei in the formation of the first cleavage spindle. The time required for this series of events varies somewhat as indicated above. In general the period is one of activity on the part of the egg. The gross form is altered constantly by pulsations though these are not as extensive as in interval 'A.' They do compare well with those of the second interval. When the whole interval is examined in detail, it is evident that there is not an immediate resumption of activity after the formation of the second polar body as after the formation of the first. Only after a considerable lag does one find the pulsations of the egg at all extensive. The peak is reached about halfway between the elimination of the polar body and the time of the appearance of the first cleavage plane. This is probably very significant, as will be shown below. Just prior to the extensive gross deformation of the egg which precedes the first cleavage, the presence of pulsations is masked by the elongation of the egg and the flattening of the poles which accompany the formation of the spindle and the appearance of the furrow. The cessation of the pulsations prior to and during cleavage is not as difficult to understand as that occurring during the formation of the polar bodies, for the former involves the entire egg while the latter involves only the polar region immediately adjacent to the maturation spindles and hence but a relatively small portion of the entire surface.

In the above descriptions only the periods designated by the intervals have been considered. Nothing has been said about the period of sperm head incorporation, polar body formation, or cleavage save that the pulsations of the egg are interrupted. The interruption is not abrupt but gradual. As the egg nears each of these stages, the outer region becomes more regular as the extent of the movements decreases. It also appears more rigid than either before or after. It has much the same appearance during the ' lag period ' which follows the isolation of the second polar body. At the time of the first cleavage, not only does the surface of the egg appear more regular but the entire egg becomes elongated in the equatorial plane in such a way as possibly to dominate or mask secondary activity. This is also true just before the appearance of the second cleavage plane.

The extent of the deformations which have been described in general above may be better appreciated upon examination of the text figure which represents tracings of a single egg during interval ' A.' It is a part of a complete record of the egg but only this period will be considered in detail inasmuch as two general types of activity are found during this period and inasmuch as the deformities at this time are of such magnitude that they are easily recognized by comparing the tracings. The period prior to and during the breakdown of the germinal vesicle is represented by nineteen tracings taken between 11 minutes and 10 seconds and 19 minutes and 40 seconds after the insemination of the batch of eggs to which this example belonged. Careful examination will show that the pulsations of the egg are of two sorts, one of which is quite extensive and results in general distortion of the sphere, and the other of which results in surface irregularities which appear more or less localized. After the ' rest period ' which ended at about 27 minutes, the egg resumed its pulsations, which may be seen to be quite extensive. These pulsations are of the more general type with no evidence of the more localized irregularities. By 37 minutes and 50 seconds and 38 minutes and 15 seconds the egg is rounding out and appears rigid as the time for the inclusion of the sperm is neared. The deformations of the intervals described above as ' B ' and ' C ' are similar to those of the second portion of interval ' A,' but in this example are not as extensive and are more difficult to follow in tracings. They are not included here.

The period between the completion of the first cleavage plane and the initiation of the formation of the second is similar to that of interval 'C.' Slow labored deformations occur which, however, are not as extensive as those noted in any of the intervals described above. They are retarded and finally cease as the blastomeres elongate prior to the second cleavage. In this connection two sets of observations may be of interest. In early experiments, the drop of sea water was not as well protected on the slides as in later ones. The evaporation of the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31 55
32 15
32 35
33:00
33:25
33:50
34:10
34 30
34:50
35.10
35 30
35:50
36:10
36:50
37 10
37:30
37:50
38:15

Deformations of the egg during interval 'A.' For explanation see text.

medium resulted in a marked hypertonicity of the solution. In such cases the eggs often did not successfully complete the first cleavage. Where no cleavage took place, the pulsations were somewhat decreased in extent even before the time of division in the controls. In addition, they ceased entirely during the cleavage period, only to reappear, though they were of lesser amplitude, after the cleavage was completed. They ceased a second time at approximately the time at which the second cleavage might have been expected. The fact that just after the cessation of the movements the eggs collapsed seems to indicate that they were approaching that stage, for it has been shown by several workers that permeability is increased at the time of cleavage. The general observations have been confirmed on eggs placed in slightly hypertonic sea water just before the first cleavage. None of the eggs were followed beyond this stage.

Discussion

Inasmuch as the above observations were made upon the relatively rigid egg of *Nereis,* it must be assumed that pulsations of this sort, found to occur in the eggs of some of the cœlenterates and described there as amœboid, are also characteristic of the eggs of many other invertebrates and probably of the eggs of some vertebrates as well. In some groups these are extensive; in others less so. In some they are evident only in the earliest developmental stages; in others they may be observed over a much longer interval. Those who are familiar with the moving picture films of fertilization and the early development of the eggs of several marine invertebrates taken by Professor Robert Chambers will recall the occurrence of movement there. The phenomenon must be much more general than might at first be thought.

In considering the portion of the egg cytoplasm responsible for the pulsations, account must be taken of both the cortical region and the deeper, more central endoplasmic mass. The localized irregularities in the surface which appear during the earlier part of interval 'A' would seem to be the result of activities in the cortical layer. It is more difficult to localize the centers responsible for the more extensive movements.

While the events described above must be significant in the development of the zygote, they apparently do not result in any immediately recognizable change in its intimate constitution. The continuous occurrence of the deformations of the egg (see below) indicates strongly that they are, to a large extent, independent of immediate nuclear control being initiated and accomplished within the cytoplasm during all phases of nuclear activity. Similar independence of nuclear and cytoplasmic phenomena has been demonstrated in a number of places, more notably by Lillie (1902, 1906) and Wilson (1904). On the other hand, the importance of a certain minimal amount of chromosomal material to the completion of cleavage has been emphasized by Dalcq and Simon (1932).

Perhaps the most interesting relationship is that existing between the magnitude of the change in shape and the varying physical state of the egg protoplasm as it has been described for numerous other forms by Heilbrunn. This worker (1928) states that the egg of *Nereis* has a relatively high viscosity when shed into sea water. This decreases greatly with the breakdown of the germinal vesicle, temporarily to increase again three times. These increases Heilbrunn associates with the formation of the meiotic spindles and of the spindle of the first cleavage. There is no mention of a fourth increase immediately after the breakdown of the germinal vesicle during what has been called the 'rest period' above, but the description is given only for comparison with the egg of *Cumingia* which has been very carefully studied. Pantin (1924), working with the unripe eggs of *Nereis diversicolor,* obtained results on the effect of temperature on viscosity which differs slightly from those of Heilbrunn for *Arbacia* and *Cumingia.*[2] It is probable, however, that the egg of the heteronereis form of *Nereis* and that of *Cumingia* are comparable. If the graph made by Heilbrunn (1921) to express the relative viscosity of the *Cumingia* egg during the pre-cleavage period be inverted, the 0 minutes be taken as 10 minutes in *Nereis,* and the time of the maturation divisions and the first cleavage fitted, the curve represents the extent of the deformations very closely with one exception. That exception is in the 'rest period' described for *Nereis* during which the pulsations cease. It is probable that the pulsations which are evident during certain phases of the development of the egg are really characteristic of all stages but are masked at certain times by the gelation of the cytoplasm of the egg as a whole. It is of interest to note that even the lag after the formation of the second polar body may be explained by reference to Heilbrunn's diagram.

It was noted above that when the eggs were developing in a medium which was hypertonic, the activity was not as extensive as in normal sea water. Similar results were obtained by Pantin (1923), who has observed that the activity of the marine amœba (*Limax* type) was reduced in hypotonic and hypertonic media.

In conclusion, the results of the observations may be summarized briefly as follows. After insemination the egg of *Nereis* shows a series of deformations which are continuous and which decrease in extent up to the time of the second cleavage. These are masked periodically by more general changes in the physical state of the entire cytoplasm of the egg, as described by Heilbrunn, so that they appear to occur in waves. It is possible that these activities on the part of the cortex culminate in the formation of the cleavage planes but at the present time the evidence does not warrant such a statement.

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[2] Costello (1934), working in Heilbrunn's laboratory, has found that the effects of temperature on the viscosity of the egg of *Arbacia* follow closely those described by Pantin for *Nereis.*

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AUTOTOMY IN THE ANOMURAN, PORCELLANA PLATYCHELES (PENNANT)

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The autotomy of parts of the individual has been observed and studied during some stage of the life history of representatives of many of the phyla of the animal kingdom. Generally speaking, animals showing this phenomenon may be subdivided into two groups, those in which the autotomy is followed by a regeneration of the lost part, and those which do not replace the missing structures. Among the latter are to be found many animals such as the echinoderms and various parasites whose adult form differs greatly from that of the larva. The first group, while not restricted to the Arthropoda, is probably best represented by that group, many of which rid themselves readily of appendages by autotomy, autospasy, or autotilly [2] for a variety of reasons. For the most part, the numerous investigators who have studied autotomy have been interested primarily in the mechanics of the process itself or in the events of the subsequent regeneration. The present observations are concerned with the extent to which an animal will cast off its appendages in response to mild stimulation, and with some apparent correlations between the sex of the individual and the amount of autotomization. Attention was first called to this aspect of the phenomenon while numbers of the small anomuran, *Porcellana platycheles,* were being examined for developing eggs.

MATERIAL

Porcellana platycheles [3] is an anomuran closely related to *Galatheinea.* The carapace of the animals used in the experiments measured approximately 8 mm. in length and 7 mm. in breadth. *P. platycheles* is a squat form with chilipeds which are broad and flattened and are drawn close to the head parts as it clings to the rocks on the lower side of which it is found. The three anterior walking legs are well developed while the fourth is slender and inflexed. The pleon is slender and is utilized in locomotion. Females bearing eggs are quite thick and have some difficulty in holding themselves to the rocks when disturbed.

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[1] This investigation was aided by a grant from the Milton Fund. I wish here to express my gratitude to the College de France for the use of space in the Laboratoire Maretime at Concarneau and to the Director, M.Réné Legèndre, and to M. E. Fauré-Fremiét, who combined to make my stay in the laboratory a pleasant and profitable one.

[2] Definitions of the methods of self-mutilation and their occurrence in Crustacea have been treated very completely by Wood and Wood (1932).

[3] I wish to thank Dr. F. C. Chace for the identification of the anomuran. An illustration of this form may be found in the *Challenger Reports* for 1873–76, *Anomura* (J. R. Henderson), Plate XI, Fig. 7. *Porcellana platycheles* and its distribution are described by Milne Edwards in "Histoire Naturelle des Crustaces," T. II, p. 255, Paris 1837.

The animals are very common on the coast of France, especially in the region south of the Manche along the shores of the Bay of Biscay. All of the animals used in the present report were collected on several small rocky islands which are located in the entrance to the outer harbor of Concarneau in Finistére. Stones and rocks in the region between the high and low tide levels were turned over and the animals found clinging closely to the roughened lower sides. They were touched and immediately dropped into a bucket in which they were transferred to the laboratory. The collection was made the last week of June, 1930, when all of the females collected save one were bearing eggs.

The appendages of *Porcellana platycheles* are autotomized upon the slightest provocation. Repeated attempts were made to preserve the animals intact for further study only to find that as soon as the animal was put into fixing fluid most of the legs were cast off. Of those which were collected and brought to the laboratory, some had lost appendages in transit. Many of the animals, however, retained all of the legs up to the time of the experiment.

METHODS

The experiments were of two kinds, both of which were very simple. Isolated animals with all of the appendages intact or with but one of the chilipeds or walking legs missing were placed in dishes in a small amount of fresh sea water and allowed to remain unmolested until they began to move freely about the container. Without moving the dish, one of the appendages was gently seized with a pair of blunt forceps and the occurrence or non-occurrence of autotomy recorded. The process was repeated with another leg and continued until each one of the appendages had been tested once. Only the chiliped and the three anterior walking legs of each side were tested inasmuch as the fourth pair is short and slender and not easily grasped without touching the owner. The chiliped of the right side was designated as 1, and the walking legs as 2, 3, and 4; those of the left side correspondingly as *A, B, C,* and *D.* Each experiment therefore involved eight separate tests.

The above experiment was varied slightly with ten of the animals. Legs were stimulated one after the other, continuing until all had been dropped. In some cases each leg was stimulated as above while in three there were repetitions before all of the legs had been tested. It was soon found that the last remaining appendages did not autotomize after

Table I

Autotomy by males. The letters and figures in Roman type indicate appendages autotomized; those in italics represent appendages retained. The two cases omitted in the averages are considered in the text.

Animal	Remarks	First 4 trials					Second 4 trials					Total 8 trials
		1	2	3	4	Total	1	2	3	4	Total	
I		C	A	B	D	4	2	*4*	*3*	*1*	1	5
III		A	2	C	4	4	*D*	1	*3*	*B*	1	5
VII		1	D	3	A	4	B	4	2	*C*	3	7
IX	Right antenna lost	D	2	A	3	4	C	4	*B*	1	3	7
X	Left antenna removed	D	4	B	2	4	A	1	C	*3*	3	7
XI	D missing	1	A	2	B	4	3	*C*	*4*		1	5 (6)
XII		1	A	2	B	4	3	C	*4*	*D*	2	6
XIII		4	D	3	C	4	2	*B*	*1*	*A*	1	5
XIV		4	D	3	C	4	2	*B*	*1*	*A*	1	5
XV		1	A	2	B	4	3	*C*	*4*	*D*	1	5
XVII		4	D	3	C	4	2	*B*	1	*A*	2	6
XVIII	A missing	*1*	*2*	*B*	*3*	0	*C*	*4*	*D*		0	0 (1)
XIX	A, 1, B missing —Large animal—	*2*	*3*	C	4	2	D				1	3 (6)
XX		1	A	2	B	4	3	*C*	*4*	*D*	1	5
XXI		4	D	3	C	4	*2*	*B*	*1*	*A*	0	4
XXII		2	B	3	C	4	1	A	*4*	*D*	2	6
Average (XVIII and XIX omitted)						4					1.57+	5.57+

numerous trials, so that the member was injured either by cutting with a pair of scissors or by crushing with forceps. Even then the leg was sometimes held for a time before being freed. The seven cases which were treated in the same way as those in the experiments described in the preceding paragraph will be considered in both connections.

EXPERIMENTS

We will consider first the results of the experiments in which each leg was stimulated but once, a total of eight successive trials. The data are presented in tabular form in Tables I and II. After a consideration of the more general aspects of the results, each of the cases which is distinguished by some qualification in the column headed "Remarks" will receive separate attention.

It is apparent after but a brief examination of the results of the experiments as they are presented in Tables I and II that there is a great deal of consistency in the behavior of the animals. Leaving for special consideration Animals XVIII and XIX, it will be seen that each of the males tested cast off an appendage at each of the first four trials. During the second four the number of the legs cast varied from 0 in XXI to 3 in VII, IX, and X. It is also of interest to note that the order in which the appendages were tested appears to have little to do with the final result. In some cases the initial trials were made with the chilipeds, in others with the fourth pair, and in still others with one of the intermediate pairs. The average of the number of legs cast by the males on the first four trials was 4, and on the second four, 1.57 +. While some of the animals will cast as many as seven legs in the eight trials, the average is between five and six.

TABLE II

Autotomy by females. The letters and figures are used as in Table II. Case VIII is considered in detail in the text.

Animal	Remarks	First 4 trials					Second 4 trials					Total 8 trials
		1	2	3	4	Total	1	2	3	4	Total	
VI		*1*	*A*	*4*	*2*	0	C	*D*	3	*B*	2	2
VIII	No eggs	B	4	D	2	4	*A*	1	*C*	*3*	1	5
XVI		*4*	D	3	C	3	*2*	*B*	*1*	*A*	0	3
XXIII		2	B	*3*	*C*	2	*1*	*A*	*4*	*D*	0	2
XXIV	Laying eggs	2	*B*	*3*	*C*	1	*1*	*A*	*4*	*D*	0	1
Average (VIII omitted)						1.5					0.5	2

Unfortunately the number of females on which records were made is relatively small. This in itself was a result of a difference in the behavior of the two sexes. As may be seen from Table II, the females do not autotomize as readily as do the males, at least while they are bearing eggs. Inasmuch as many of those with developing eggs were preserved before the experiments were begun, fewer remained for the actual tests. Those preserved did not drop more than one or two of the appendages until placed in the fixing fluid. Even then they generally retained some of their members. Disregarding for the moment the results obtained with Animal VIII, the only female examined which did not bear eggs, it will be seen that the females as a group cast from 0 to 3 legs on the first four trials and from 0 to 2 on the second four. The average for the first four is 1.5 and for the second four is 0.50. The average for the eight is 2. Only one of the females autotomized any appendage in the second period of the experiment and there the number cast was two. These averages, which are to be found in Table II, should be compared with those for the males in Table I. The difference in the behavior of animals of opposite sexes must be significant.

We will now consider those cases which have been qualified in the table.

Nothing need be said of Animal XI save that the third walking leg of the left side was missing when the experiment was begun. The total number of legs cast before the animal ceased to react was therefore 6 instead of 5, as would appear from the record. Number XIX lacked 3 of its legs but was tested because it was an unusually large animal. During the trials three more of the legs were dropped, which brings the total in that case up to 6 in place of the 3 which appear in the table. Inasmuch as so many of the legs were missing when the experiment was begun, it was considered legitimate to omit this case from the averages made and presented above. Female XXIV was laying eggs at the time the experiment was performed. This animal autotomized but one leg and that on the first trial.

One male (IX) was tested from which one of the second antennæ was missing. As has been mentioned above, the flagellum of the antenna is very long and hence is easily injured. The animal cast 7 of its legs in eight trials, failing on the seventh. The next animal experimented upon was also a male, so that the left second antenna was removed by scissors before the legs were touched. That animal (*X*) also dropped 7 of its legs, failing only on the eighth trial. Inasmuch as Animal VII also dropped 7 of its legs and a number dropped 6, no other experiments of this sort were made.

Of all of the females collected and examined only one (VIII) had no eggs attached. Not only did this animal bear no eggs, but its behavior in autotomy was similar to that of the males. It is possible that an error was made in sexing the animal, which, unfortunately, was not preserved, but that seems highly improbable. It is possible that there was some functional disorder present which resulted in the absence of the ova. It is also possible that the animal had already laid its eggs and that all of the young were hatched prior to the time of collection. Inasmuch as the rest of the females bore eggs which were far enough along in their development to show eye spots and yet not old enough to be hatched, this does not seem probable. The animal was not included in computing the averages.

Male XVII is another case in which an animal showed an autotomy reflex which was characteristic of the opposite sex. The left chiliped was missing when the experiment was begun. No appendages were cast during the seven trials which were made on the remaining members. The behavior, therefore, was characterized by more inhibition to autotomization than is generally found in the female. It is probable that the absence of the autotomy reflex should be explained on some of the grounds to be discussed below. If the animal had recently molted, for example, autotomy would not be expected. In that case, however, the absence of the left chiliped must be attributed to autospasy or combat, and not to true autotomy. Inasmuch as the reactions of the animal were so evidently atypical, it is not considered in connection with the averages above.

The second type of experiment involved the successive stimulation of appendages, one after another, until all or all but one had been autotomized. So far as possible, the data are summarized in Table III. Three sorts of information will be found embodied in the figures. They indicate the number of trials made on any specific leg and the number of the trial (in the total number) at which the leg autotomized. They also indicate whether the leg was dropped in response to the mild stimulation employed throughout in the experiment above or in response to injury either by cutting or by crushing. Occasionally a leg is retained even after injury and later released, apparently in response to mild stimulation (I, II). On the other hand, in three of the cases (VI, IX, X) injured members were held to the end of the experiment. Seven of the cases reported here are also to be found reported in a slightly different way among the experiments of Tables I and II.

It is immediately apparent on examination of the table that the females tend to retain the legs longer than do the males. This is evident not only in the larger number of attempts required to cause all of the legs to be released but also in the number of legs which have to be injured before they are autotomized. It is also evident, both in the

TABLE III

Autotomy on repeated stimulation. In the columns headed "Trial on which the leg was cast," the subscripts indicate the number of trials with that leg. Where no subscript is present it is understood that the leg was cast on the first trial. In the column headed "Injury," the subscripts indicate the number of the trials at which that particular leg was injured.

Animal	Remarks	Trial on which the leg was cast									Total trials	Sex
		1	2	3	4	A	B	C	D	Injury		
I		11_2	5	18_5	17_6	2	3	1	4	3_5 4_5	18	♂
II		28_4	17_2	25_5	33_8	16_4	18_3	36_9	6	A_4 C_7 1_4	36	♀
III		6	2	28_{10}	4	1	25_8	3	17_5	B_8 D_5	28	♂
IV	Both antennæ removed	26_8	6	10	8_2	25_{11}	7	9	5	A_{11} 1_8	26	♂
V	Twelve trials	—	4	—	5	—	3	—	—	A dropped in Bouin's	—	♀
VI	B eight trials but not cast	22_4	18_3	7	6_2	16_4	—	5	17_2	A_4 B_7 D_2 1_4	25	♀
VII		1	7	3	6	4	5	11_4	2	C_4	11	♂
VIII		1	4	16_4	2	17_4	1	15_4	3	A_4 C_4 3_4	17	♀
IX	B eight trials but not cast	8	2	4	6	3	—	5	1	B_5 B_7	15	♂
X	3 eight trials but not cast	6	4	—	2	5	3	7	1	3_6	15	♂

males and the females, that a resistance to autotomy is built up as repeated trials are made and more and more of the legs are cast. Two males (IX, X) and one female (VI) failed to autotomize the last remaining leg even on repeated stimulation after injury. As in the data of the previous experiment, the record of animal VIII shows its behavior to be much more like that of a male than a female. Unfortunately, animal XVIII was not employed in this series.

DISCUSSION

The general question of autotomy in the decapod Crustacea has been discussed in considerable detail in a recent report by Wood and Wood (1932). Those desiring an account of the earlier work on this subject should consult their paper. The observations of these investigators cover a wide variety of forms found both on these shores and at Roscoff. Unfortunately they were unable to obtain *Porcellana* but they state concerning *Galathea squamifera*, a closely related anomuran, that it "autotomizes on less stimulation than any other form used." While they were interested primarily in the mechanics of autotomy, a number of their observations are of importance to the present discussion.

Porcellana casts its appendages by the method described by Wood and Wood as true autotomy. They distinguish between three types of self-mutilation, all of which are dependent upon the physiological state of the animal for their realization. The following observations are mentioned specifically here as they may have some bearing on the results obtained above. Animals kept in the laboratory for some length of time, even though apparently in good condition, may fail to show the typical autotomy reflex (*Cambarus* var. sp.). Loss of blood may so weaken an animal that in spite of a vigorous pinching response by the chilipeds, no typical autotomy can be induced. Experiments on *Carcinides moenas,* the green crab, have shown that newly molted animals are not able to autotomize their appendages. *Carcinides* infected with *Sacculina* autotomized as readily as did uninfected animals. No sex differences in autotomy were observed.

Turning now to the information obtained in the experiments reported above, it is quite apparent from Table I that animals with all of the appendages present autotomize much more readily than do animals which have already cast some of their legs. The act of autotomy in itself appears, therefore, to have a very definite influence on the reflex. There are two possible explanations for this fact which immediately come to mind. The animal may be in a poorer condition after the shedding of some of its legs so that it resembles in its reactions individuals which have been bled or have been held in the laboratory. On the other hand, the autotomy itself may alter the typical reaction of the animal by freeing some substance or substances which act as inhibitors to the process. This possibility should be tested. It is of interest to note that while the first legs to be cast are released after mild stimulation, extensive injury is often insufficient to cause the last legs to be dropped. The inhibitory influence must be a potent one.

The order in which the appendages are stimulated and dropped has little or no effect upon the total number which will be cast by an individual. When the experiment is begun with the anterior appendages, the two posterior walking legs will usually remain, while if the posterior walking legs are the first to be tested, the two chilipeds are retained. One complete experiment of the first type required much less than five minutes, so that the factor or factors responsible for the change in the reflex must act very quickly. Inasmuch as the legs are freed by true autotomy it may be assumed that the act is accompanied by very little loss of blood. The evidence does not favor a control by inhibition due to the lowered "vitality" of the animal.

A single test was made with an animal which had previously lost more than one of its appendages. This was a large male with both of the chilipeds and the first walking leg of the left side missing. Each of the five remaining members was tested once with the result that three of them were autotomized. The total number of legs cast by the animal was therefore six, which compares very favorably with the total number dropped in eight trials by intact animals. If the cause for the retention of the last legs is to be found in the production of some substance which modifies the reactions of the individual, either this animal must have lost the three members just before the beginning of the experiment or the effects of the autotomy of the limb must persist for some time. The results obtained with those animals from which one (IX and X) or both (IV) of the second antennæ were missing when the experiment was begun have a distinct bearing on this question. Mutilation of one antenna apparently has no effect on the behavior in autotomy though mutilation of two may have such an effect. Were the change in the reflex the result of loss of blood, it might be expected that injury to the antennæ would be as potent as injury to a leg in this connection. The evidence in the experiments does not confirm this hypothesis. The effect appears to be intimately associated with the loss of the legs themselves.

Both of the tables prepared in connection with the experiments contain definite evidence that the behavior of animals of the two sexes differs in respect to autotomy.[4] Males cast more of the legs than do females carrying eggs and in addition they cast the legs more readily than the females. It is unfortunate that the number of the females examined was so small but, as stated above, the primary interest in the females carrying eggs led to the preservation of many before the experiments were commenced. There is such a high degree of consistency in the results obtained that the relatively small number may be taken as representative of the group. The difference in the behavior of the two sexes might be attributed to a structural difference which would make the reflex mechanically difficult. Wood and Wood (loc. cit.) have stated that animals with heavy exoskeletons do not easily accomplish true autotomy. No difference in the nature of the carapaces was noted here. The presence of eggs produces changes in the gross relations between the parts of the body which might be thought to be significant. Wood and Wood have shown, however, that *Carcinides* infected with *Sacculina* can autotomize its appendages. The relationships in that case would be similar to those in animals bearing eggs. The parasite is found on the males but as the same workers have shown, there is no difference in the mechanics of the process in the two sexes. Moreover, the very fact that animals of both sexes will drop some of their legs by true autotomy shows that the reflex may be accomplished by both.

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[4] M. R. Legèndre has informed me that he has noted similar sexual differences in *Porcellana platycheles*.

The difference in the behavior of the males and the females carrying eggs is more satisfactorily explained on the assumption that there is some fundamental difference in their functional status which modifies the reflex. It is well known that the sexes differ as to their metabolism. Moreover, in some Crustacea, animals of opposite sexes have dissimilar pigmentation during the breeding season (shrimps).[5] In the Insecta sex differences in behavior are found in the termites and the ants where the female autotomizes the wings at the conclusion of the nuptial flight. Similarly, some of the parasitic flies lose their wings when they reach the host on which they take up their permanent abode. The alteration or differentiation of the behavior pattern according to the mode of life or the sex of the individual is a very common occurrence. The evidence in the present case points to a similar fundamental difference in the reactions of the two sexes in *Porcellana.* It would be very interesting to know the behavior of females at other seasons of the year. Some indication might be inferred from female VIII, but this is an isolated case and should not be taken as representative of the group without further experimentation.

The discussion would not be complete without some brief reference to the data obtained in relation to adaptation. Autotomy itself has been considered from this standpoint so often that nothing further need be said here. The behavior of the females in relation to the males is of interest for during the breeding season the female is less active than the male and hence less liable to injury. Moreover, the gravid female is continually dependent upon her appendages both in the care of the eggs and in defense, while the mode of life of the more active male changes but little. Fortunately the male, which is more subject to injury, autotomizes more readily than the female, which makes constant use of its legs.

LITERATURE CITED

Wood, F. D., and H. E. Wood, II, 1932. Autotomy in Decapod Crustacea. *Jour. Exper. Zoöl.*, **62**: 1.

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[5] I am indebted to Dr. Frank A. Brown for this information.

EXPERIMENTAL PROCEDURES IN A STUDY OF THE LOCATION AND CONCENTRATION WITHIN THE HOST CELL OF THE VIRUS OF TOBACCO MOSAIC

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It has long been felt by many investigators that the active agent in any typical mosaic disease of plants may be intimately associated with the protoplasm of the affected host. This view is no doubt due in part to the mass of evidence indicating rapid multiplication and migration of the virus in the living tissues of the host, and the lack of convincing evidence that such virus agents may be propagated *in vitro*. It is quite conceivable that the virus agents of many animal diseases reach body fluids and secretions, and ultimately find ready means of transfer to other host individuals. Since in plants there are no such general fluids, with the exception of that of the "transpiration stream," it seemed particularly desirable and important to direct efforts toward the determination of the location and concentration of the virus agent in the various parts of the living cell, including, of course, the various protoplasmic components and the vacuole. It appears that no such study has previously been attempted. The "typical" mosaic of tobacco offered many advantages and was selected as the main object for the preliminary studies here reported.

Methods and Materials

Micro-methods

A Chamber's micro-manipulation apparatus, consisting of two manipulation units and a Leuer syringe attachment for extraction, was used in this work. The operations were carried out under a Spencer microscope equipped with a mechanical stage, using a 4 mm. objective and a 6 × ocular, giving an approximate magnification of 260 ×.

The micro-pipettes were made from hand-drawn capillary Pyrex tubing with an approximate inside diameter of 0.5 mm. and a wall thickness of approximately 0.1 mm. The tips of the pipettes were drawn out to a fine point with a fairly abrupt taper, ending in a tip with an opening of from 0.5 μ to 1. μ in diameter. It was found necessary to have

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[1] This work was made possible, in part, through a grant from the Research Committee, Graduate School, University of Wisconsin

the taper fairly abrupt and uniform to the very tip in order to prevent clogging and to facilitate withdrawal of the cell contents. Inasmuch as the type of pipette suited for this work was very difficult to make by hand, a mechanical device was designed which produced very uniform pipettes of the type required. This device, which embodies certain modifications of the principle utilized in an earlier machine designed by Du Bois,[2] will be described in a subsequent paper (Livingston, 1934, in press). The tips of the pipettes were bent upward at an angle of 15 to 20°, sealed into the special adapters with DeKhotinsky cement, and attached to the tube leading from the syringe, which was mounted on one of the manipulator units. In those cases in which a larger opening in the tip of the pipette was desired for some of the work, the tip was broken off under the microscope with a needle attached to the other manipulator, leaving a jagged opening with a diameter of 2 or 3 μ.

After considerable experimentation, 20 per cent gelatin was used as a mounting medium. This was used because of the following advantages it possessed over the usual liquid mounting media: (1) It holds the tissue firmly in place, so that it is easy to puncture and to enter the cell to be operated upon at any point desired, this firmness being unattainable in liquid media with the tissue used in these investigations. (2) Since the gelatin is solid, there is no danger of the mounting medium entering the pipette and diluting the extracted material. (3) The low solidification point of the gelatin, which is slightly above room temperature, makes it possible to apply it in a liquid state without danger of injuring the tissue by excess heat.

Because the hair cells of the tobacco plant are readily accessible and are sufficiently large to be easily operated upon, and are known to contain a high concentration of the agency causing the mosaic disease, these cells were used in this study. Freshly diseased shoots from young, rapidly growing plants were used, and thin cross-sections were cut with a sharp razor from the base of the midrib of a diseased leaf or from the first internode of the stem above the lowest leaf showing definite mosaic symptoms. These sections were mounted on a 24 x 30 mm. cover slip in a drop of gelatin as described above. Several sections were cut at one time, and the mounted material was kept in a closed Petri dish on moist filter paper until operated upon. Except where specified below, no material was used in these studies which had been mounted more than 6 hours. Over this period of time, the cells remained fully turgid and exhibited active streaming.

For operation upon the cells, the cover-slip with the mounted material was inverted over a moist-chamber and was held in place by a coating of Lubriseal. It was found that cooling the tissue markedly

[2] *Science* (N. S.), **73**: 344–345, 1931.

increased the viscosity of the protoplasm, which greatly facilitated certain operations. When this was desired, the material was thoroughly chilled on ice directly before inverting the cover-slip over the moist-chamber, and the moist-chamber was kept surrounded by a stream of brine from an ice-salt mixture throughout the operations.

As it was impossible to measure directly the quantity of material withdrawn, the portion of the pipette which was filled in the operation was carefully measured with a micrometer ocular, and the cubical contents computed. For each run, from 0.15 to 1 cu. mm. of material was collected, and this was diluted with distilled water to the concentration desired for inoculation. As it was impossible to expel all of the material from the pipettes, and the possibilities for adsorption were very great with such minute quantities, the tips of the pipettes were broken off and ground in a micro-mortar in the water used for dilution in order to free as much material as possible.

All inoculations were carried out by a standardized needle-prick and scratch method, using healthy potted plants grown in the greenhouse. As a control, thoroughly macerated hair cells, from the same diseased plant used for the micro-manipulation work were diluted to a concentration of 1/1,000 and inoculated in the same manner.

Macro-methods

It seemed probable that an application of Chibnall's[3] technique for securing separately samples of vacuolar sap and of protoplasmic extract might be made useful in the present study, and although recognizing the limitations of such macro-methods, this procedure was modified to suit our requirements.

The tobacco shoots infected with the virus of typical tobacco mosaic were grossly washed of any adhering dust or soil, dried of surface water under an electric fan, dipped and held in ether 3 minutes, and then immediately placed in an hydraulic press and subjected to a pressure of about 2,000 lbs. per square inch of plunger area. It is recognized that, while some of the cells are ruptured by this process and doubtless some unfiltered sap is thus obtained, it is also true that some sap is obtained which is filtered through protoplasm and cell wall under pressure. In the experiment referred to a volume of 30 cc. of juice, designated vacuolar sap, was obtained. This was later diluted (sample A) for the inoculations, as indicated in Table I. The remaining tissue residue was ground finely twice in a food grinder, 70 gm. were recovered, and this was diluted with 250 cc. distilled water, in order better to extract the virus. The residue was then pressed at 4500 lbs., and the "infective" juice collected was brought up to a volume that would yield a concentration of approximately 1/10. From a part of this juice (sample B) the other required dilutions were prepared. Another part of this 1/10 juice (sample C) was also treated with diatomaceous earth [4] and then diluted for inoculation as in the case of sample D.

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[3] *Jour. Biol. Chem.*, **55**: 333–342, 1923.

Shoots of young tobacco plants infected with the virus of yellow mosaic were treated in precisely the same manner, as in the case of the typical mosaic, for the preparation of corresponding samples of this virus. All inoculations were carried out by a standardized needle-prick and scratch method, using potted plants grown in the greenhouse.

Results and Discussion

Some Physical Properties of the Cell Inclusions and the Relation of These Inclusions to the Concentration of the Virus Agent in the Cell

Although numerous cytological investigations have been made on the cell inclusions accompanying various virus diseases, micro-manipulative technique offers not only a new approach to the problem of sampling, but also in the present studies has made possible certain interesting observations on the physical properties of these cell inclusions.

There are two recognized types of intracellular bodies or inclusions which normally accompany the typical mosaic disease of tobacco, viz., the vacuolate type, sometimes designated as amœboid or X bodies, and the " crystalline " type or " striated " bodies. Both types are usually definitely associated with the cytoplasm of the cell. In certain of the hair cells, both types may be found; in others, they may occur separately. In the material used in the present studies, the " crystalline " bodies appeared to be much more numerous than the vacuolate type, and if the vacuolate type were present, they were usually accompanied by one or more of the crystalline type. As the " crystalline " bodies are often profoundly altered in appearance by the action of cytological fixatives, they are frequently overlooked in studies made on fixed and stained material.

The presence of either type of inclusion in the cell seems to bring about a rather marked decrease in the viscosity of the cytoplasm. It was noted that the cytoplasm of hair cells from a diseased plant which had no inclusions present, and which appeared to be normal in every respect, was sufficiently viscous so that on cooling to slightly above 0° C. it would not readily enter a pipette with an opening in the tip of slightly more than 1 μ in diameter. This was particularly true of the outer region, or the so-called ectoplasm. In contrast, the cytoplasm of cells with abundant inclusions would readily enter a pipette with a similar opening, and there seemed to be no noticeable differences in the viscosity of the various regions of the cytoplasm, regardless of cooling or of any other treatment resorted to. The phenomenon of semi-liquefaction of the cytoplasm would perhaps be expected in cells in an abnormal or pathological condition, such as that attending the presence of the virus agent and the formation of the inclusions.

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[4] Duggar, B. M., *Proc. Soc. Exper. Biol. and Med.*, **30**: 1104–1109, 1933.

The vacuolate inclusions appear to be surrounded by a more or less definite membrane or surface film which, for the time at least, maintains them in a definite form and preserves their identity. Ordinarily they will withstand considerable disturbance in the cytoplasm in their immediate vicinity, but if touched by a micro-needle or by the tip of a micropipette they break down immediately into a kind of granular mass, which readily enters a pipette with an opening of 1 μ in diameter. This observation seems significant.

The so-called crystalline bodies normally appear as flat, irregular, plate-like crystals, which, when seen on edge, often show a striated or palisade-like structure. Although in the living cell they often appear to be definitely crystalline in nature, they break down into a granular mass immediately on being touched or disturbed by the tip of a micropipette, and they readily enter the opening of the smallest pipette used. This behavior indicates that they are not truly crystalline.

Inasmuch as certain investigators have hypothecated that the inclusion bodies are actually concentrates, or at least an indication of high concentrations, of the virus within the cells in which they occur, it was deemed desirable to investigate the comparative concentration of the virus agency in hair cells from diseased plants which possessed abundant inclusions, in contrast with those cells, either from the same plant or from comparable plants, which possessed no visible inclusions of any kind. Accordingly, a young plant which had been inoculated 15 days previously, and which had shown definite mosaic symptoms for approximately one week, was selected, and sections were cut from the stem above the lowest leaf showing definite symptoms. These sections possessed abundant inclusions (both crystalline and vacuolate types) in the hairs in certain regions, while on other regions of the same section no inclusions were present (the former may correspond to the paler areas of leaves, the latter possibly to the greener areas). The sections were mounted as previously described, and were operated upon at ordinary room temperature, using pipettes with an opening of 2–5 μ in diameter. Under these conditions it was possible to extract the entire cell contents, and material was collected from hair cells with abundant inclusions, as well as from hair cells on the same plant which possessed no visible inclusions. As a control, an inoculum was prepared from hair cells clipped from the same diseased plant. All inoculations were made at a dilution of 1/1,000 on young, rapidly growing plants, using 10 plants for each series.

As may be noted on reference to Table I, *A*, the material from cells possessing abundant inclusions induced 80 per cent disease at the dilution used, while the material with no inclusions induced no disease. The controls were all diseased, as is usually the case in experiments carried out at a dilution of 1/1,000 with the technique used in these experiments. The slight diminution of the incident disease in the material collected from cells with abundant inclusions over the control is ascribed to adsorption and other difficulties involved in the micromanipulation technique.

TABLE I

Indications of the relation of inclusion bodies to the concentration of the virus agency within the cell.

	Material collected	Dilution for inoculation	No. plants inoculated	No. plants diseased	Percentage diseased
	mg.				*per cent*
A.					
Cell content (inclusions present).	0.249	1/1,000	10	8	80
Cell content (inclusions absent).	0.303	1/1,000	10	0	0
Control (macerated hairs)......	3.0	1/1,000	10	10	100
B.					
Cell content (inclusions present).	0.577	1/100	3	3	100
		1/1,000	4	4	100
Cell content (inclusions absent).	1.064	1/100	5	1	20
		1/1,000	10	0	0
Control (macerated hairs)......	2.0	1/1,000	10	10	100

For the purpose of further checking the results indicated in the above experiment, the test was repeated, collecting sufficient material to inoculate at a dilution of 1/100 as well as at 1/1,000. Diseased material from a later series, but of an age and condition similar to that used in the experiment described above, was used in this series. All sections employed possessed hairs with and without inclusions. However, as it was impossible to collect sufficient sap in one day's time to complete the experiment, different diseased plants were used as sources of virus for the various parts of this series, although the diseased material was from the same lot and in every way comparable. From the cells with inclusions present, 0.577 mg. of the cell contents was collected, an amount which, on dilution, was sufficient to inoculate 3 plants at a dilution of 1/100 and 4 plants at 1/1,000. As indicated in Table I, *B*, all plants inoculated developed the disease. From the cells with inclusions absent, 1.064 mg. was collected, which, on dilution, was sufficient to inoculate 5 plants at a dilution of 1/100 and 10 plants at 1/1,000. Of these, one plant inoculated at 1/100 developed the disease, while the 1/1,000 series was entirely disease-free. The control, which consisted of hair cells collected from the same plant used for the material without inclusions, but which possessed abundant inclusions in various areas, induced 100 per cent disease.

The results of these experiments might indicate either that the inclusions are actual concentrates of the virus agency, or, as seems more reasonable, that they represent products accompanying the development of the agency in high concentrations. Inasmuch as these structures are intimately associated with the protoplasm of the cell, the suggestion may be offered that the protoplasm itself is the place of origin and development of the agency causing the mosaic disease of tobacco.

Preliminary Indications on the Location and Concentration of the Virus Agent within the Cell

Indications from Macro-methods.—As previously indicated, a modification of the Chibnall technique was used in these studies which were made on both typical tobacco mosaic and on the yellow mosaic of tobacco.

The results shown in Table II seem clearly to indicate that the sap pressed from the tissues with as little disintegration of the protoplasm as is possible with this technique contained the virus only in relatively low concentration. It should be remembered that ordinarily the incidence of disease at a dilution of 1/1,000, with the inoculation technique employed, averages around 90–100 per cent. On the contrary, the virus is found in the residue at full strength. Admitting the filtration possibilities of both protoplasm and cell walls, the results are nevertheless suggestive, and it was the more important to seek evidence from micromanipulation (the micro-pipette method).

Indications from Micro-methods.—In these studies an attempt was made to remove the cell sap from diseased cells in as pure a state as possible and to contrast the infectivity of the cell sap with that of the entire cell contents from similar cells. In this way it was hoped to determine whether the virus agency was localized in the protoplasm or if it was present in high concentrations throughout the cell.

It must be recognized at the outset that any dissection or disturbance within the cell may bring about profound changes in its organization and condition, resulting in disturbances in adsorption and similar phenomena which might tend to seriously alter the results obtained. However, by exercising care in the operation, and by working as rapidly as possible, these tendencies were no doubt greatly minimized, and while the results may not be strictly quantitative, they at least will serve as indications of the relative concentration of the virus agency within the various regions of the cell.

For the removal of the cell sap, only freshly mounted, thoroughly chilled hair cells from plants affected with "typical" tobacco mosaic were used. It was found, as indicated above, that it was important that the material used be in good physiological condition in order to effect fairly accurate fractionation. Accordingly, shoots from plants which were just beginning to show definite mosaic symptoms, having been inoculated ten days previously, were used in this experiment. The sections were cut from the first internode above the lowest leaf showing definite mosaic symptoms and were mounted as previously described. Approximately one-third of the hair cells possessed inclusions, chiefly of the crystalline type, although the cytoplasm of these cells still possessed sufficient viscosity to allow for fairly accurate fractionation of the cell sap versus cytoplasm if the material was kept at the proper temperature. The openings in the tips of the pipettes used did not exceed 2 μ, since openings of a larger size would allow a certain amount of cytoplasm to enter, regardless of other precautions. The pipettes were quickly thrust into the center of the cell, the cell sap withdrawn chiefly

TABLE II

Indications from macro-methods as to the location of the virus agency within hair cells of tobacco plants affected with "mosaics."

Sample	Dilution for inoculation	Typical mosaic diseased	Yellow mosaic diseased
		per cent	*per cent*
Vacuolar sap, natural juice	1/1	100	100
	1/10	80	20
	1/100	50	0
	1/1,000	0	0
	1/10,000	0	0
Extract from tissue residue, natural juice	1/100	100	100
	1/1,000	90	90
	1/10,000	80	70
Extract from tissue residue, celite treated juice	1/100	100	100
	1/1,000	70	100
	1/10,000	40	40

by the capillary attraction of the pipette, supplemented occasionally by very mild suction applied to the syringe, and then quickly withdrawn. If any appreciable amount of cytoplasm entered the pipette under these conditions, it would be readily detected by the difference in refractive index and the behavior in the pipette, and the entire pipette would be discarded. Several pipettes were used in the extraction of the material, because the pipettes quickly lost their capillary action as the tips became filled, and often a small bit of cytoplasm would adhere to the tip and clog the opening.

The entire cell contents were removed from cells of similar plants with essentially the same technique described in the previous section, that is, operation on the cells at room temperature, and using pipettes with a larger opening. Hair cells were clipped from the plant used in the removal of the cell sap, macerated, and diluted 1/1,000 as a control.

TABLE III

Indications from micro-methods as to the relative concentration of the virus agency in the cell sap and in the cytoplasm of hair cells from diseased tobacco plants

	Material collected	Dilution for inoculation	No. plants inoculated	No. plants diseased	Percentage diseased
	mg.				*per cent*
Cell sap	0.577	1/1,000	10	2	20
Cell contents	0.581	1/1,000	10	8	80
Control	2.5	1/1,000	10	10	100

The results of a typical experiment (see Table III) indicate that the main concentration of the virus agency is in the protoplasm of the cell. The degree of infection resulting from the cell sap inoculations (20 per cent disease) may be due to the unobserved entrance of a small amount of cytoplasm or to the disturbance of the cytoplasm by the injury to the cell, resulting in the freeing of the virus agency into the cell sap. However, final conclusions on this matter must be withheld until it is possible to make a more thorough study than has been possible to date.

SUMMARY

In this paper experimental evidence is offered which strengthens the view that the virus of typical tobacco mosaic occurs primarily, if not solely, in the protoplasmic components of the cell, rather than in the vacuole. From the observations made it would seem clear that the occurrence of intracellular bodies, in hair cells at least, is coincident with or an accompaniment of relatively high virus concentration. The inclusion bodies, both vacuolate and striated types, are fragile structures disintegrating on contact with the micro-needle or pipette.

FURTHER STUDIES ON THE LONGEVITY AND SWIMMING ABILITY OF SPERMATOZOA

BENJAMIN H. GRAVE

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A series of experiments designed to measure the total distance which spermatozoa of *Cumingia tellinoides* and *Arbacia punctulata* are capable of swimming, the rate at which they progress, the period of their greatest activity, and their longevity, are described in this paper. They were carried out at the Marine Biological Laboratory, Woods Hole, Massachusetts, during the summers of 1931 and 1932.

My indebtedness to Dr. M. H. Jacobs, Director of the Laboratory, for the use of a room and for many conveniences, is gratefully acknowledged.

Apparatus and Methods

Following the publication of a preliminary paper on this subject in 1928 (*Jour. Exper. Zool.*, vol. 51), work was continued with refinements in methods and apparatus used. The construction of the sperm tube as finally perfected is shown in Fig. 1. It may be noted that a slender feed tube (*FT*), inserted at each end, makes it possible to introduce sperm at one end of the tube and eggs at the other and that this method of introducing the sperm reduces to a minimum any disturbance of the water. The elevation (*E*) in the floor of the tube at either end is designed to prevent eggs and sperm from drifting. The two open tubes (*VT*) in the top are intended for ventilation but are probably unnecessary.

In setting up an experiment, the sperm tubes are first filled with sea water which has stood in jars upon the laboratory table over night and which therefore has assumed the temperature of the room and is free from any gases it may have acquired while under pressure in the pipes of the laboratory water system. The sperm tube is then immersed in water of the same temperature with only the feed tubes and ventilating tubes projecting above the surface. For this purpose rectangular aquaria filled with sea water which had also stood on the table over night were used. This undoubtedly insures a uniform environment and avoids convection currents or other disturbing movements of the water as far as possible.

After the sperm tubes, so filled and immersed, had stood undisturbed not less than two hours, one or two drops of eggs were introduced in the feed tube at one end of each sperm tube and two or three drops of a 1 or

2 per cent suspension of sperm at the other.[1] Both eggs and sperm were seen to settle quietly in the feed tubes and to drop vertically from their inner ends, each into the appropriate pocket. It is evident, therefore, that if any of the eggs in the tubes ultimately undergo cleavage they must have been fertilized by spermatozoa that have traversed the full length of the tube. By using tubes of different lengths it was possible to determine not only how far the spermatozoa are able to swim but also the rate of their locomotion.

A possible fault of the former experiments, as reported in 1928, may be found in the fact that the tubes then used were exposed to air and that convection currents might thus be set up within the sperm tubes during the course of the experiment which might therefore have carried the spermatozoa farther than they normally swim of their own effort. However, by carefully shielding the tubes from environmental changes, including air currents, almost the same results are obtainable by either method. By employing an improved piece of apparatus, and by immersing the tubes in water, this source of error has been eliminated.

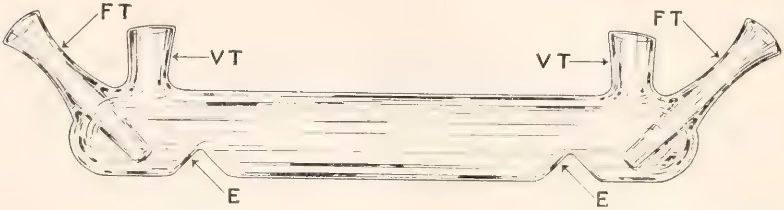

FIG. 1. Type of sperm tube used. *FT*, feed tube; *VT*, ventilating tube; *E*, elevation in the floor of the sperm tube.

METHOD OF CALCULATING THE RATE OF SWIMMING OF SPERMATOZOA

The average normal rate of cleavage of *Arbacia* and *Cumingia* eggs at temperatures of 23 or 24° C. is approximately as follows: first cleavage, 45 to 50 minutes; second cleavage, 70 to 75 minutes; third cleavage, 95 to 100 minutes. By deducting cleavage time from the total elapsed time of the experiment the swimming time of individual spermatozoa for traversing each sperm tube is closely approximated.

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[1] Although the initial concentration of the sperm suspensions used in these experiments was relatively high, it became diluted as soon as the spermatozoa began to spread into the water in the body of the sperm tube. The generally recognized influence of the concentration factor on the longevity of sperm may be considered negligible here.

RESULTS

During the summers of 1931 and 1932 numerous experiments of the type just described were made in which approximately constant water temperatures in the sperm tubes were maintained at 22, 23, 24 and 25° C. The time required for spermatozoa to traverse the tubes was repeatedly tested. The results, after the method was perfected, were entirely consistent both in rate of swimming and distance attained. Although spermatozoa were shown to swim for periods of 9 or 10 hours, an unexpected aspect of their activity became evident in that their rate of progress after about 2 or 2½ hours slows down suddenly. A further gradual slowing continues from this point to the end of the life of the sperm. During the latter and greater part of the swimming period the spermatozoa are probably quiescent most of the time, swimming only intermittently. Such intermittent activity, alternating with periods of inactivity, satisfactorily accounts for the observed slowing down in the swimming rate at about the 2-hour interval. These observations on the most active period of spermatozoa are in substantial agreement with other data on the longevity of gametes. Former experiments made to test the longevity of spermatozoa of *Cumingia* and *Hydroides* (*Biol. Bull.*, vols. 54 and 59) have shown that almost 100 per cent fertilization of eggs may be obtained from dilute suspensions of spermatozoa 3 hours old; a small percentage live and are capable of fertilizing eggs 9 to 12 hours after emission, and very few live and function for longer periods.

A comparison of experiments on the gametes of *Cumingia* and *Arbacia* shows that the spermatozoa of the former are somewhat more vigorous than the latter. The spermatozoa of *Arbacia,* with one exception, were observed to swim but 15 to 16 cm. while those of *Cumingia* usually swam 15 to 18 cm.

Results published in a former paper gave the swimming ability of sperm of both species as 28 or 30 cm. It now appears that the extreme limit for even the most vigorous *Cumingia* sperm is 20 cm. Experiments, when performed in tubes exposed to air, frequently give the results originally published. The unexpected difference in the distance covered by spermatozoa in tubes immersed in water and those surrounded by air must be accounted for as the effect of convection currents set up in the water by the more variable temperature environment afforded by air.

Differences in the rate of swimming, as computed from data of recent experiments, show that the spermatozoa of each species are variable in their activity, probably due to differences in vigor. Table I gives the composite results of a series of experiments on the rate of swimming of spermatozoa of *Cumingia* at the temperatures indicated.

The rate at which spermatozoa of *Arbacia* swim these distances are not greatly different from that given in the table. They slow down more quickly than the sperm of *Cumingia* and may require 8 or 9 hours to swim 16 cm. and 4 or 5 hours to swim 15 cm. The rate at which the first 12 cm. is covered is about the same for sperm of the two species. There is a perceptible but not great difference in the rate of swimming of sperm of both species at temperatures of 22 and 25° C. The rate is somewhat slower at the lower temperature and a reduced total swimming distance is also observed. No experiments were made at temperatures lower than 22° C. because of lack of adequate constant temperature apparatus, experience having shown that any considerable change of temperature during the course of an experiment vitiates the results.

EXPERIMENTAL DATA

From the twenty-one experiments made during the summer of 1932, one (No. 18) is selected for description as typical. None of these experiments included less than five sperm tubes and some involved as many as twenty, the tubes varying in length from 7 to 18 cm. Each experiment required from 5 to 9 hours for completion. All of the tubes of an experiment were stocked with sperm from a single individual and with eggs of a single lot. Controls of unfertilized eggs were kept to make sure that they did not cleave because of chance fertilizations. All experiments showed consistent results although the final ones were the most successful because of improvement in technique with experience. Detailed descriptions of additional experiments would show nothing essentially significant or different from those selected.

TABLE I

Rate of swimming and distance attained by spermatozoa of Cumingia at temperatures of 23 to 25° C.

Length of Sperm Tube *cm.*	Time Required to Swim the Distance
7	40 to 50 minutes
9	50 to 65 minutes
12	1¼ to 1¾ hours
15	1½ to 2½ hours
16	2½ to 5 hours
18	5¼ to 9 hours

Experiment 18. July 27, 1932

Sixteen sperm tubes of various lengths were set at 12:00 M. Two drops of sperm and two drops of eggs of *Arbacia* were added to each tube. The records of six of these tubes are given as follows:

Tube No. 1. Length, 7 cm.; temperature, 24.5° C. Eggs examined at 1:40 P.M. Result: Five eggs just cleaving into two cells. Total time of experiment, 100 minutes; less cleavage time of 45 minutes, swimming time 55 minutes. Sperm swam 7 cm. in 55 minutes.

Tube No. 2. Length, 12 cm.; temperature, 24.5° C. Examined at 3:10 P.M. Result: two eggs in 2-cell stage and one in 8-cell stage. Total time, 190 minutes; cleavage time required to reach 8-cell stage, 94 minutes; swimming time, 95 minutes. (Sperm therefore swam 12 cm. in 1 hour and 35 minutes.)

Tubes No. 3 and 4 were also 12 cm. in length but were kept at a temperature of 22.5° C. (Aquarium placed on a cool water table.) Sperm traversed them in 130 and 135 minutes respectively.

Tube No. 5. Length, 15 cm. Temperature, 24.5° C. Examined at 4:30 P.M. Result: One egg in 4-cell stage, three in 8-cell, and three in 16-cell stage. Total time, 4½ hours. Time required to reach the 16-cell stage, 120 minutes; swimming time, 150 minutes. (Sperm therefore swam 16 cm. in 150 minutes.)

Tube No. 6. Length, 18 cm. Temperature, 24.5° C. Examined at 8:40 P.M. Result: Four eggs in the two-cell stage. Total time 8¾ hours. Swimming time 8 hours.

Comment: This experiment shows that the rate of swimming of the sperm slowed down promptly after 2½ or 3 hours and this was apparent in the whole series of experiments. It also shows that they swam somewhat more rapidly at a temperature of 24.5° C. than at one of 22.5°.

This is the only experiment of the series in which sperm of *Arbacia* swam more than 16 cm. It is probably not exceptional.

Experience has shown that spermatozoa may swim from 2 to 3 cm. after the fifth hour or between the fifth and tenth hours. They make little or no progress after 9 hours.

Discussion

A repetition and reëxamination of the former work extends the data to include the rate of swimming as well as the total distance spermatozoa are capable of swimming and shows that their vigor is rapidly reduced after a relatively brief period of active swimming.

The sharply marked reduction in rate after 2 hours of swimming, as shown by Table I, is significant. It probably means that although spermatozoa live and function for much longer periods, their vitality and energy are practically spent within 2½ hours. There seems also to be a great difference in the vitality of the sperm of different individuals of the same species.

The first spermatozoa to arrive at the opposite end of the sperm tubes and therefore the first to fertilize eggs have been considered as giving the rate of swimming. As a matter of fact, larger numbers of spermatozoa arrive later, and others continue to arrive for several hours, as shown by the fact that certain 12 cm. tubes which were allowed to remain undisturbed for 10 hours contained every stage of cleaving eggs from two cells to the blastula. This also shows that spermatozoa continue to swim more or less for a period of 9 or 10 hours.

Spermatozoa which have been stimulated to great activity and weakened by hours of swimming may become quiescent and lie almost motionless. If these spent spermatozoa are now brought into the presence of eggs, or of sea water which has contained eggs for some time, they often revive and again swim actively. It is possible that spermatozoa are attracted by emanations from the eggs and that they are directed in their swimming by chemotaxis. The conditions of our experiments make it unlikely that stimuli act through any considerable distance, and it may be assumed that we are measuring only the activity of spermatozoa as stimulated by normal sea water. If, therefore, the direction of swimming of the spermatozoa is at random and without orientation it is probable that a spermatozoön which crosses from one end of a sperm tube to the other in the course of several hours may actually have travelled a greater distance than the length of the tube, especially if it reversed the direction of its locomotion several times in the process. There is no way to ascertain or measure this sinuous path. Hence the length of the tube is taken as the distance attained. Ignoring these complications, it is concluded that vigorous spermatozoa of certain marine invertebrates may swim 20 cm. in sea water and that they attain three-fourths or four-fifths of this distance during the first 3 hours after extrusion into sea water.

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ON THE REALITY OF SPINDLE FIBERS

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It is a striking fact that our knowledge of chromosome structure and behavior is far in advance of what we know about the rest of the mitotic figure. In spite of the interest of earlier workers in the mitotic spindle, we have very little definite information about it and as proof of this may be mentioned the fact that there is still disagreement on such a basic point as to whether the so-called spindle fibers have a morphological reality in the living spindle. It is this latter question with which I am here concerned.

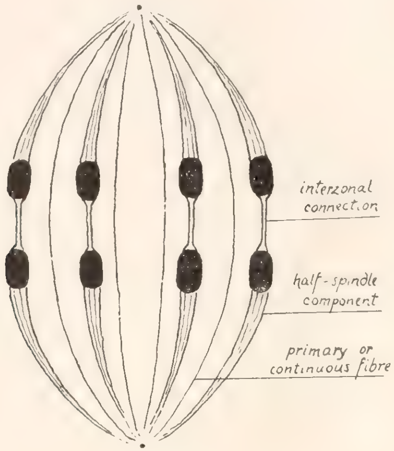
For the sake of clarity it may be well to indicate the structures that are involved. They were recognized by some of the earliest cytologists and though their exact nature may not be clear, it is nevertheless patent that on a topographical basis alone one may distinguish three types of fibers in the fixed anaphase spindle: (1) The primary or continuous fibers that usually extend from pole to pole and do not establish any contact with the chromosomes; (2) the half-spindle components each of which is connected with a chromosome on the one hand and one of the poles on the other; (3) the interzonal connections, which stretch between the separating daughter chromosomes (text fig. 1).

The Present Status of the Question

That the spindle as a whole is morphologically differentiated from the general cytoplasm has been amply demonstrated by such work as that of Chambers and Sands (1923) and is indeed quite evident from an examination of the living cell. The question at issue is whether the fibrous structures seen in fixed preparations have some morphological representation in the living spindle, or whether the latter is quite homogeneous.

Naïvely expressed, it is evident that something must be responsible for the structures seen in fixed spindles. So far as I can see, only two alternative explanations are possible. One is that spindle fibers represent lines of force which, although invisible in the living spindle, bring about a differential coagulation when the latter is fixed. Such a coagulation results in the longitudinal streaks or lines that we call fibers. In other words, the proponents of this view maintain that in the unfixed spindle there is no morphological basis for such fibers. In contradistinction, the alternative view is that the living spindle is not homogeneous and that the fibers seen in fixed cells are present in some morphological form also when the cell is alive.

However, the issue is complicated by the fact that some early and much recent evidence indicates that the spindle is not to be regarded as a unit and that, as I have argued in an earlier publication (1932), the three spindle elements are not necessarily related to each other in any basic way. Since the material used in the present investigation is not favorable for work on the primary or continuous fibers, I will venture no conclusion concerning them. On the other hand, the interzonal connections can hardly be regarded any longer as a subject of the question here involved, since their actuality now seems sufficiently well established. In substantiation I need mention only the work of Němec (1929 and earlier), who was able to separate the phragmoplast from the nuclei by centrifuging and other means and still found the orthodox fibers appearing in it on subsequent fixation; the report of Ellenhorn (1933) that interzonal connections (which, like me, he interprets as tubes) are visible in certain living anaphases; and finally, the persistence of interzonal fibers to the metaphase and anaphase of the succeeding division (Schrader, 1932).



TEXT FIG. 1

But no such definite evidence is available for the half-spindles and it is to them that my main attention is directed. Speaking generally, the case for a homogeneous structure of the half-spindle is based on the three following arguments:

1. In the normal, living cell the half-spindle shows no fibrous structure and appears perfectly homogeneous.

2. Chambers (1924) reports that when a microdissection needle is inserted into the half-spindle region of a living cell and moved to and fro, no corresponding movements occur among the chromosomes. Again, if a chromosome is pulled out of the metaphase plate it shows no fiber adhering to it. He argues that this must indicate the absence of any morphological connection between chromosome and pole.

3. Some cytologists, especially certain members of the school of Gregoire, believe that after good fixation spindle fibers appear in the stained preparation no more than they do in the living spindle.

The first of these arguments may well be admitted as a weighty one. So far as I know no one has ever demonstrated conclusively that fibers may be seen in the living spindle. I can only suggest, as many have done before me, that the indices of refraction of fibers and ground substance may be so close to each other that an optical distinction becomes impossible under ordinary conditions.

The evidence brought forward by Chambers and so frequently referred to, can hardly be considered as decisive. As Bělař (1929*a*) has already pointed out, we know almost nothing about the resistance that might be offered to the needle by the fibers. Nor do we have any grounds to believe that a fiber pulled out into the cytoplasm is a conspicuous object. At best, Chambers neglected to take the obvious step of fixing and staining the cells thus operated upon. Their appearance then would, of course, be of great importance.

The claim that in perfectly fixed cells, and especially those at the periphery of the tissue, no spindle fibers appear, may well be taken seriously when it is advanced by such careful workers as Robyns (1929 and earlier) and Bleier (1930 and 1931). But the claim is not easily put to a test. It must be remembered, however, that good fixation does not necessarily imply favorable staining properties. That peripherally located cells are fixed more perfectly than more deeply lying ones is almost universally accepted, but I would like to point out that such cells frequently stain imperfectly. Thus the cells at the periphery of the heteropteran testis are frequently more rapidly destained than those nearer the center; and in the case of such Amphibia as *Amphiuma, Batrachoseps,* and *Plethodon,* these peripheral cells very often never accept such stains as saffranin and hæmatoxylin at all. That applies especially to chromosomes in the prophase stages as well as the spindle elements, and of the morphological reality of the former there can, of course, be no doubt. In short, a good fixation does not in itself guarantee the appearance of all cell organs after staining, the latter depending on additional factors, chief of which appears to be the hydrogen ion concentration (Seki, 1933).

To sum up, the evidence against a structural reality of half-spindle fibers is not complete. But a similar criticism has been made of the case for the other side of the question. The evidence there, usually indirect, is exemplified by such investigations as those of Bělař (1929*a* and *b*) and Schaede (1930). These workers produced, the one by immersion in hypertonic media, the other by centrifuging, splits in the spindle. These splits always occur in or parallel to the long axis and both investigators concluded that, conservatively speaking, there must be a structural differentiation, longitudinally oriented, in the living spindle. To this Bleier (1931) has objected that if through such external agencies splits arise in a homogeneous body under bipolar tension, they must of necessity take the general direction of such tension and do not indicate a preëxisting longitudinal structure per se. He makes similar criticisms of some others of Bělař's ingenious arguments and though I do not concede that he completely disposes of the latter, it is evident that the case is still open.

In view of this rather unsatisfactory status of the question, it is strange that so little attention has been paid to a type of evidence that has been in the literature for some time. This evidence pivots on the simple argument that if spindle fibers can be bent in the living cell, they can not be interpreted simply as lines of force. Such bending has been described as resulting from natural or at least unknown causes by Bonnevie (1906) and others; it has been observed as resulting from the cell distortions involved in the effects of hypertonic media (Bělař); and most frequently of all it has been reported as consequent on the centrifuging of dividing cells (among others, Andrews (1915), Lillie (1909), Morgan (1910), Spooner (1911), and Schaede (1930)).

It will be recognized at once that the bending of the half-spindle fibers becomes significant only if it can be established that the experimental treatment had not already induced coagulation when the bending occurred. This implies that controls similarly treated must show that such cells continue to live and divide. Such controls are manifestly impossible in naturally occurring distortions. The effects of hypertonicity are not conclusive because Bělař states that, whereas anaphases may recover, metaphases so treated almost never do. Unfortunately, it is the latter in which we are certainly concerned with half-spindle fibers, and therewith the objection becomes possible that the fibrous structure reported by Bělař was first produced by the hypertonic medium and then bent and distorted by continued action of the latter.

The evidence from centrifuging suffers because none of the workers distinguish sharply between half-spindle fibers and interzonal connections and, indeed, most of their figures deal with the latter. However, Lillie speaks of serious disarrangement of fibers and shows in Fig. 7 of his paper a metaphase which is not very striking, and Morgan remarks on and shows in Fig. 32 of his paper a slight bending of the half-spindles in a metaphase. The best evidence is contained in the paper by Spooner, who reports that spindle fibers may be bent without permanent injury to the cell and illustrates this effect especially in Fig. 3 of her paper, where two metaphases show bending of the half-spindles.

I can conceive of only one loophole in the argument. If the surface of the spindle body is, let us say, negatively charged, and the hypothetical lines of force are under its influence, then a bending of the spindle surface might possibly result in a corresponding deflection of the interior lines of force. The consequent fixation would then form fibrous coagulations which, in conformation to the lines of force, would be bent. If, however, the centrifuged spindle shows fibers that are not only curved but in addition show some displacement with respect to each other, this last objection would fall to the ground. Miss Spooner's figures are too sketchy to allow of a judgment on the point and I therefore undertook the rather thankless task of once more covering the ground.

MATERIAL AND METHODS

An Emerson centrifuge with 10,000 revolutions per minute and an 8 cm. radius was used throughout. The centrifuge has the advantage of reaching full velocity within 3 or 4 seconds and coming to a full stop within the same length of time.

The following material was used:

Heteroptera: testes of *Alydus eurinus*, *Brochymena arborea*, and *Euschistus fissilis*. Fixation: strong Flemming.

Mollusca: eggs of *Planorbis glabratus* in first and second cleavage. Fixation: Bouin.

Crustacea: testes of *Homarus americanus*. Fixation: strong Flemming. Eggs of several species of *Cyclops* in early cleavage stages. Fixation: Carnoy or Carothers.

Heidenhain's hæmatoxylin method was used in staining and all preparations were mounted in Euparol.

HETEROPTERA

Testes of *Alydus* were centrifuged ½, ¾, 1, 2, and 3 minutes; testes of *Brochymena* and *Euschistus* for 1, 2, 3, 5, 7, and 9 minutes. One testis of each specimen was fixed immediately after centrifuging while the other was retained as a control in modified Ringer's solution. The remarkable result was that none of the metaphases even when centrifuged for 9 minutes showed a significant distortion. The interzonal fibers of the anaphases, on the other hand, already showed bending and twisting effects at 3 minutes. This fact, of course, can only emphasize that a distinct difference must exist between the two spindle regions. It was found, however, that whereas uncentrifuged testes remain normal in the Ringer's fluid for 7 or 8 hours, testes centrifuged for 5 minutes or longer already show evidence of degeneration after about four hours. Such cells show a characteristic granular deposit in the cytoplasm and frequently small vacuoles make their appearance. Deleterious effects are thus produced in material centrifuged for such periods, even though in material fixed immediately no detrimental results are to be observed.

Mollusca

After centrifuging (from 1 to 3 minutes), the egg masses of *Planorbis* were divided into two portions, one to be fixed at once and the other to be kept as control in ordinary pond water. In spite of the stratification of the egg materials, development occurred normally in all the centrifuged controls, as Morgan and Conklin have already reported in other mollusks. However, metaphase spindles were only sporadically affected by centrifuging, the cases of bent half-spindles occurring with about equal frequency for all the periods used. Apparently such bending results only when a mass of yolk globules becomes accidentally lodged on one side of the spindle and it is therefore not the direct result of centrifuging. In no such instance was the effect very striking (Fig. 4).

Crustacea

Homarus

From testes with spermatocyte divisions portions approximately half a centimeter long were cut out, the controls being derived from the topographically corresponding region of the other branch of the testis. The material was centrifuged for ½, 1, 1½, 2, and 2½ minutes. Controls

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Plate I

1. *Homarus.* Normal metaphase of spermatocyte division.

2. *Homarus.* Spermatocyte metaphase centrifuged for 1½ minutes, showing two broken components in the lower half-spindle and a chromosome with its components dislodged on the right side.

3. *Homarus.* Spermatocyte metaphase or early anaphase showing extreme distortion after being centrifuged for 1½ minutes.

4. *Planorbis.* Metaphase of first cleavage in the egg, centrifuged for 2¼ minutes. Note yolk globules on left side of spindle.

5. *Cyclops americanus.* Telophase of third cleavage in egg centrifuged for one minute, showing distorted interzonal connections.

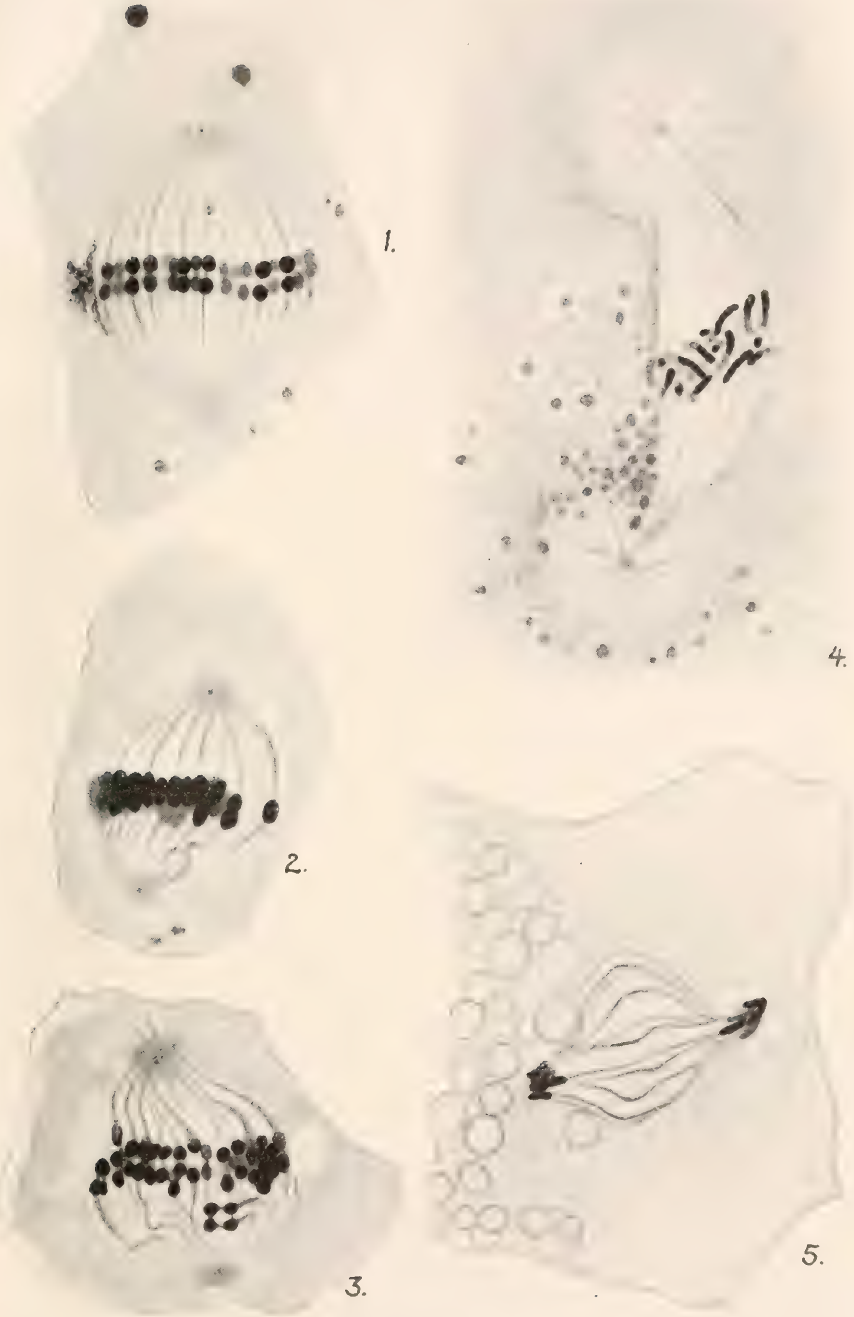

PLATE I

were kept in Ringer's fluid or in sea water sterilized with heat. I found it impossible, however, to avoid bacterial action which generally set in after 5 or 6 hours.

Mention must be made of the fact that spindle fibers of uncentrifuged spermatocyte cells show a certain amount of crinkling, the direct effect of fixation shrinkage. But this effect is readily distinguished from the more extreme distortion resulting from centrifuging.

Centrifuging for 1½ minutes produces effects such as are shown in Figs. 2 and 3. It will be seen that not only are the fibers distorted, but they are shifted with respect to each other and not infrequently torn out of the main body of the spindle. The case of *Homarus* would thus be decisive in establishing an actuality of half-spindle fibers if the cells involved would through recovery attest that coagulation had not occurred. Unfortunately this can not be definitely established. Controls examined three hours after centrifuging, indeed, show that in many regions the cells appear normal, but in other, rather sharply restricted regions of the same tissues a definite degeneration is evident. The distortion effects already described are found in similarly restricted regions and I can only suggest that we are dealing here with the results of a crumpling and folding within the narrow confines of the centrifuge tube. It is very likely the pressure incident to such folding that is to be held responsible for such effects and of course also for the later degeneration. But in view of the likelihood that degeneration occurs in cells with bent spindles, the material becomes useless for our purpose and was reluctantly abandoned.

Copepoda

The two egg masses carried by females of various species of *Cyclops* are almost ideal for the purposes of the experiment. In the two species used, each mass contains about a dozen eggs and these, at least during early cleavage, are all in practically the same phase of mitosis. The egg masses easily can be detached from the mother's body and continue to develop normally in ordinary pond water.

In centrifuging, both egg masses of a female were used, one being fixed immediately afterward while the other was kept in pond water for various lengths of time before fixation. All the drawings were made from *Cyclops americanus,* which was readily obtained in the spring. But with the beginning of June this species became scarce and for purposes of studying the effect of centrifuging on the time of mitosis, *Cyclops signatus* was used.

It should be remarked that here, as in *Homarus* and *Planorbis,* the effect of centrifuging seems to be an indirect one. If the spindle, as sometimes happens, lies parallel to the stratification of yolk and other materials, it appears to be affected very little. But usually it has a diagonal position so that one pole becomes pushed into or against the stratified yolk while the other is under pressure from the peripheral portion of the egg. It is chiefly in such cases, which comprise the great majority, that the effects to be described are encountered.

The normal, uncentrifuged metaphase of *C. americanus* is shown in Fig. 6. The distance between the poles of the early cleavage figures is unusually great. The half-spindle fibers are sharply marked and stain rather heavily for a part of the distance between the chromosomes and the pole, but then they suddenly become fainter and can hardly be traced near the tip of the spindle. The astral center appears to be at some distance from this tip. The fully formed half-spindle as a whole is plainly demarked from the cytoplasm and though it is difficult to show in drawing, there appears to be a matrix or ground substance which occupies the spaces between the fibers or components.

Centrifuging for one minute frequently produces distortion of the metaphase spindle, but the effect is more or less sporadic. The interzonal connections of the anaphase are, however, nearly always distorted in such eggs, the result depending on the position of the mitotic figure at the time of centrifuging. Thus the group of interzonal fibers may be bent as a whole if the two groups of chromosomes are shifted with respect to each other, or else they may be kinked and separated if the centrifugal force coincides with the long axis of the spindle in its direction (Fig. 5). Centrifuging for 1¼ and 1½ minutes causes similar if not greater effects on interzonal fibers, but it also is more constant in its effects on the half-spindles. Since centrifuging for 1¾ minutes did not markedly increase such changes, nearly all the observations were made on eggs centrifuged for 1¼ or 1½ minutes.

It will be remembered that in the early divisions of the copepod egg there is a striking gonomery. This side by side association of maternal and paternal nuclei in a double spindle is easily disturbed by centrifuging,—one spindle often being shifted on the other. But generally this is done through bending of the poleward or distal region of the half-spindle without severing connection with the asters. This region seems to offer little resistance, whereas the more sharply outlined parts of the half-spindle components proximal to the chromosomes are evidently stiffer (Figs. 7 and 10). Occasionally, however, the two spindles become completely separated at one pole, although in my experience in every such case the connections with the opposite pole were maintained.

In two or three instances the spindles show a splitting or separation of the components without any marked bending. Such a phenomenon has already been described by Bělař (1929) and more particularly

Schaede (1930), and will not be especially mentioned in the present work (Fig. 10).

It is with the more common cases of distortion that I am particularly concerned. That the half-spindle fibers are relatively stiff has already been remarked, but bending nevertheless occurs even in the proximal region. It is to be emphasized that the components of a half-spindle are not only bent as a group, but that bending occurs unequally so that even adjacent ones may show varying degrees of distortion and displacement (Figs. 7, 8, and 9). However, it is rarely that a fiber or component is so dislodged as actually to protrude from the general spindle body, a result which is so common in *Homarus*. It must be obvious that no system of lines of force would show reactions of the type just described.

The possible criticisms of such a demonstration of the actuality of spindle fibers have already been referred to. To check the possibility that centrifuging first coagulates a homogeneous spindle substance in fiber-like streaks and then, being continued, produces the bending here described,—centrifuged metaphases were studied in vivo. If the visibility of fibers depends on coagulation in a homogeneous substance, and if such coagulation can be induced by centrifuging, then fibers should be seen in freshly centrifuged, unfixed metaphases. The spindles are found without much difficulty, but though they are studied more readily than uncentrifuged figures (because centrifuging removes the obscuring yolk spheres), it is not often that an exact side view is encountered. In none of the four instances where this was done could any definite fibrous structure be identified and except for the absence of yolk the appearance did not seem to differ from that of uncentrifuged spindles. Evidently, a coagulation of the ordinary sort is not involved.

A more extensive study was made of the effects of centrifuging on subsequent activity of the egg. In normal and uncentrifuged eggs of *Cyclops signatus* it takes very close to three-quarters of an hour to com-

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Plate II

(All figures of *Cyclops americanus*. In Figs. 6, 8, and 9 only one member of the double spindle appears)

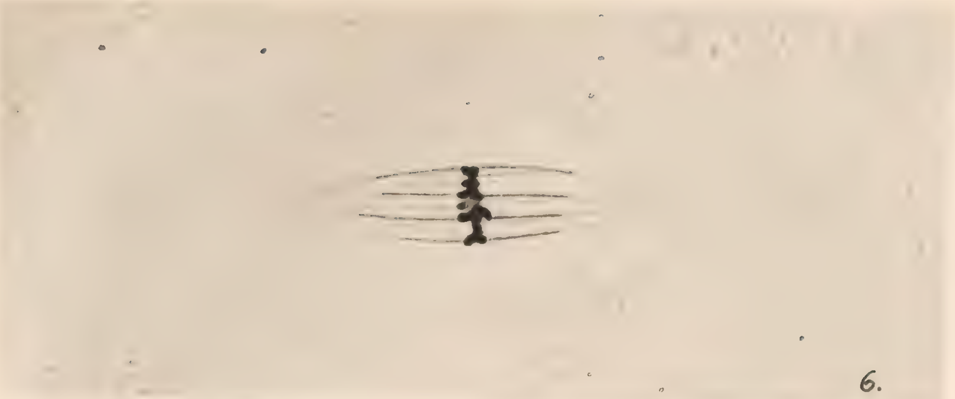
6. First cleavage metaphase in normal, uncentrifuged egg.

7. Second cleavage metaphase, centrifuged for 1¼ minutes. Showing a shifting of the two gonomeric spindles on each other, with compensating bends in the half-spindles near the poles.

8. First cleavage metaphase, centrifuged for 1¼ minutes. Showing that half-spindle components can be bent independently of each other.

9. First cleavage metaphase, centrifuged for 1¼ minutes. Showing that half-spindle fibers can be bent independently of each other.

10. Second cleavage metaphase centrifuged for 1¼ minutes. Showing splitting in one of the gonomeric spindles and bending of the polar end of the half-spindle.



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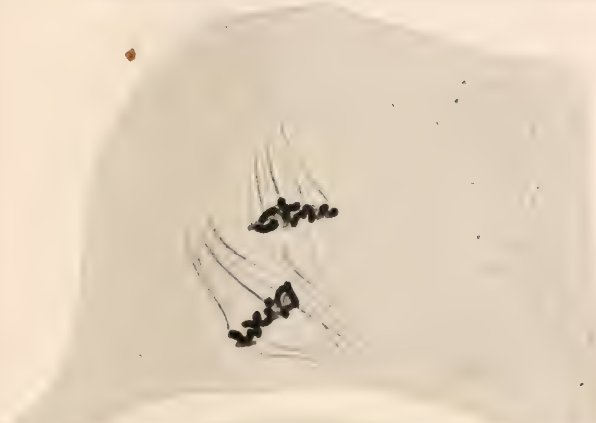

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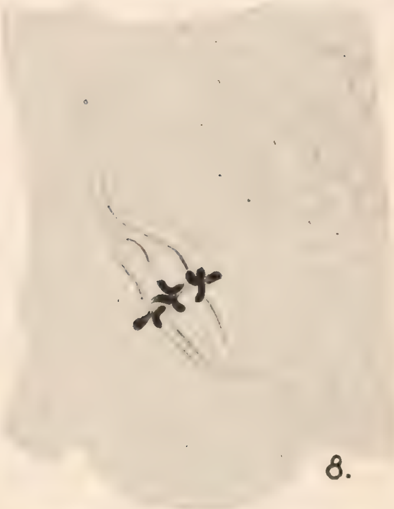

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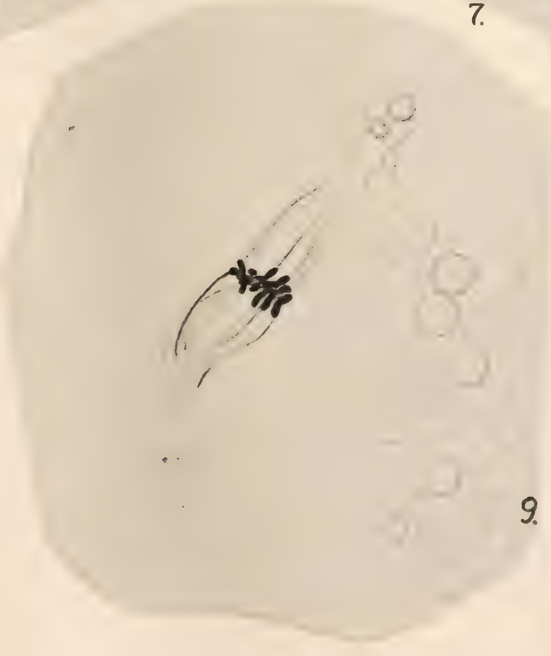

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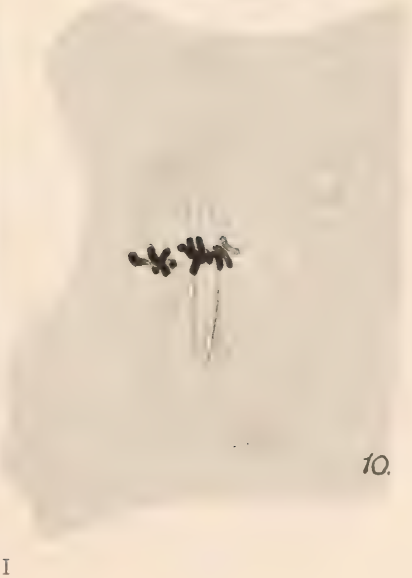

10.

PLATE II

plete an early cell cycle, i.e., from metaphase to metaphase. Eggs centrifuged when in the first or second metaphase and fixed one-half hour afterward had reached the middle prophase of the succeeding division (12 eggs); after three-quarters of an hour the eggs had reached an early metaphase (with a formed spindle but some trace of the disintegrating nuclear membrane) of the division following (11 eggs); after one hour an early prophase of the second following division had been reached (6 eggs); after 2 hours the succeeding two divisions had been completed and the prophase of the third had been initiated (about 20 eggs); and after 5 hours eggs centrifuged when in the first metaphase had attained the stage in which blastula formation is begun (about 30 eggs).

It will be seen that the duration of a cell cycle in eggs centrifuged during first or second metaphases is thus not markedly different from that in uncentrifuged eggs, that is, about three-quarters of an hour. The possibility of some slowing nevertheless remains, not only because it is difficult definitely to limit the various phases but also because the metaphase appears to be of some duration and it is not possible to recognize whether one is dealing with one that has just been established or one that is about to be terminated for the anaphase. But this period does not exceed 10 minutes at best and is more likely to be in the neighborhood of 5 minutes. A lag due to centrifuging thus cannot be greater than a few minutes.

In short, the centrifuging either brought about no delay in the mitotic activities or else the latter were caused to lag for only a negligible space of time. This again must mean that no coagulation is involved, and this in turn implies that the fibrous or at least longitudinal structures were present in the living half-spindle.

Conclusion

Throughout the preceding pages the word " fiber " has frequently been used. It must be understood that it is employed for convenience only since there is every indication that so far as the half-spindle is concerned the structures involved have a greater complexity than the term " fiber " would indicate. Indeed, the same may be said for the interzonal connection although, as I have tried to show in a previous paper, the half-spindle and interzonal elements show some very definite differences from each other. The case for such a difference certainly becomes stronger in the light of Ellenhorn's recent observations and finds still further support in the present investigation, where the relative ease with which the interzonal connections are distorted in the Heteroptera and Copepoda stands in distinct contrast to the behavior of the half-spindle components under identical experimental conditions.

However, so far as the present work is concerned, the detailed structure of these elements is not involved. To repeat, the question here is simply whether the living spindle is homogeneous or whether there is a morphological basis for the half-spindle components, as they appear in fixed preparations. I consider that the evidence can point only to the last-named of these alternatives.[1]

I must concede, however, that I have not been able to overcome an objection that has been expressed in connection with the argument of Bělař and Schaede, that longitudinal splits in the treated spindle presuppose a corresponding structure in the normal spindle. This objection is voiced by Bleier (1931) as follows (freely translated): " From such splits one could more logically deduce that the main spindle is composed of smaller spindles (Teilspindeln)." It so happens that the spindles of copepod eggs are particularly open to this objection, if such it be. I adduce this not only from direct observation (especially of the maturation figures) but also from the readiness with which the chromosomes are separated from each other experimentally, each accompanied by a tiny spindle (Schiller, 1909). The close relationship of such spindle structure to that seen in *Acroschismus* (Hughes-Schrader, 1924), where the compound nature is evident in normal, untreated spindles, is too obvious to merit further comment.

But whether these small, component spindles are called Teilspindeln or whether they are called half-spindle components as I have done, seems to me of little import. That they are more firmly linked together in some forms than in others, is clear, but even in such a closely knit spindle as that of the Heteroptera the similarity of half-spindle components to tiny individual spindles is so striking as to raise the question whether all spindles are not basically composed of Teilspindeln. It is true that in many, perhaps most, cases the finished metaphase spindle seems to present still another and homogeneous substance which occupies the spaces between the half-spindle components, but if this is present in such cases as *Acroschismus* and *Llaveia,* it must arise quite secondarily.

Indeed, if Bleier could commit himself more definitely to the implication in the statement quoted, he and I might well find ourselves on common ground—to wit, that the half-spindle is primarily composed of smaller elements which have hitherto been called fibers but which I have termed more noncommittally " components."

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[1] It is of interest to note that Belling apparently was also convinced of the reality of the half-spindle components, for referring to some unpublished work he wrote (1933, p. 80): ". . . 'Traction' fibers of the spindle are real, I consider, in *Lilium.* I have, by starving, got them to show in a vacuole."

Unfortunately nothing beyond this short statement is given. It is hoped that the manuscript pertaining to the work in question is in condition to be published.

SUMMARY

1. By centrifuging, the fibers or components of the half-spindle are bent and distorted, not only as a group but also independently of each other.

2. Metaphases so treated continue their mitotic activities and it is concluded that the half-spindle components as seen in fixed preparations are not coagulation artefacts but have a morphological basis in the living spindle.

3. The experiments lend further support to the claim that half-spindle components and interzonal connections differ from each other in structure and certain other physical properties.

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OBSERVATIONS ON THE GENUS STERRHURUS LOOSS, WITH A DESCRIPTION OF STERRHURUS BRANCHIALIS SP. NOV. (TREMATODA, HEMIURIDÆ)

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The genus *Sterrhurus* was erected by Looss (1907*a*) with *S. musculus* as the type species. In the genus he included also *S. imocavus* Looss, *S. grandiporus* (Rudolphi, 1819) [= *Dist. grandiporum* Rud., = *Lecithochirium grandiporum* (Rud.) Lühe, 1901], and *S. fusiformis* (Lühe, 1901) [= *Lecithochirium fusiforme* Lühe]. Looss included the genera *Sterrhurus, Lecithochirium* Lühe, 1901, *Synaptobothrium* von Linstow, 1904, *Plerurus* Looss, 1907, and *Brachyphallus* Odhner, 1905 in the subfamily Sterrhurinae. Fuhrmann (1928) listed *Synaptobothrium* as a synonym of *Lecithochirium* and transferred *Brachyphallus* to the subfamily Hemiurinae. Recently, Manter (1934) added the new genera *Dinosoma* and *Parasterrhurus* to the subfamily Sterrhurinae.

In a more extended paper, Looss (1907*b*) discussed the morphology of the hemiurid trematodes and their taxonomic relations. He gave a detailed account of the characteristic features of the genus *Sterrhurus,* and brief specific diagnoses of the four species listed above. Subsequently seven other species have been added to the genus. Two of them, *S. monticellii* and *S. lævis,* had been described by Linton (1898) and assigned to the genus *Distomum. D. monticellii* was described from the stomach and gills of *Remora remora* and later Linton (1901) also reported the parasite from the intestine of *Pomatomus saltatrix* and *Paralichthys dentatus.* Linton (1905) reported both immature and gravid worms, all of which were listed tentatively as *D. monticellii,* from nineteen different species of fish at Beaufort, North Carolina. Often only a single specimen, and in other cases only a few of them were found in each host. The species was recorded in his (1907) studies on the parasites of Bermuda fishes, and, in his account of the trematodes of the Dry Tortugas, Linton (1910) placed it in the genus *Sterrhurus.* Linton (1898) also described *Distomum læve* from *Macrourus bairdi.* Looss (1899) classified this worm as *Hemiurus lævis* (Linton) and Manter (1931) transferred it to the genus *Sterrhurus.* Another species, *Sterrhurus brevicirrus,* was described by Nicoll (1915) from the intestine of *Pomadasis hasta.* Manter (1934) redescribed *S. lævis* (Linton) and added the new species, *S. floridensis, S. præclarus, S. ro-*

bustus and *S. profundus.* Nigrelli and Stunkard (1933) reported a species of *Sterrhurus* from the gills of the cutlass fish, *Trichiurus lepturus* Linn. Further study of this material shows the specimens to be specifically distinct from all previously described forms and for them the name *Sterrhurus branchialis* is proposed.

Sterrhurus branchialis sp. nov.

More than three hundred sexually mature worms were taken from the gills and seven others were found in the intestine of a single individual of *Trichiurus lepturus.* The fish was taken near Long Island, New York, although the natural habitat of the species is the deep water of the warm Atlantic, extending north to Virginia and occasionally as far as Massachusetts.

Fixed and stained specimens are 0.76–2.5 mm. long and 0.34–0.57 mm. wide. The body is only slightly flattened, wall strongly developed, smooth when the specimen is fully extended but thrown into numerous fine annulations when the worm is contracted. The cuticula in the anterior part of body is 0.003–0.009 mm. in thickness. The " tail " is much narrower than the body proper, when fully extended about one-third the body length. It is filled with homogeneous, chromophile globules (presumably excretory products) which in living worms were observed to pass to the exterior through the pore at the tip of the " tail." The acetabulum is 0.2–0.4 mm. in diameter, situated 0.26 to 0.72 mm. from the anterior end, depending on the degree of contraction in this portion of the body. The dorsal lip is present and may show two or three weakly-muscular lobes or papillæ.

Digestive System. The oral sucker is 0.11–0.16 mm. in diameter. The pharynx is immediately behind and about three-fifths as large as the oral sucker. The esophagus is short; intestinal rami extend to the posterior third of the body and enter the " tail " only when the animal is strongly contracted. The distal ends of the ceca are slightly dilated.

Male Reproductive System. The testes are somewhat lobed, spherical to oval, the long axis lateral in contracted specimens, 0.11–0.235 mm. in diameter. They are postacetabular, usually oblique, sometimes symmetrical (especially in immature, small, and contracted individuals). Ducts from the testes unite dorsal to the acetabulum to form the S-shaped seminal vesicle. The initial portion of the vesicle is large, saccate, followed by the anterior curved portions. The anterior part is dorsal to the sinus sac and a short recurved duct, the pars prostatica, connects the seminal and prostatic vesicles. The terminal half of this duct is widened and opens at an angle into the prostate vesicle.

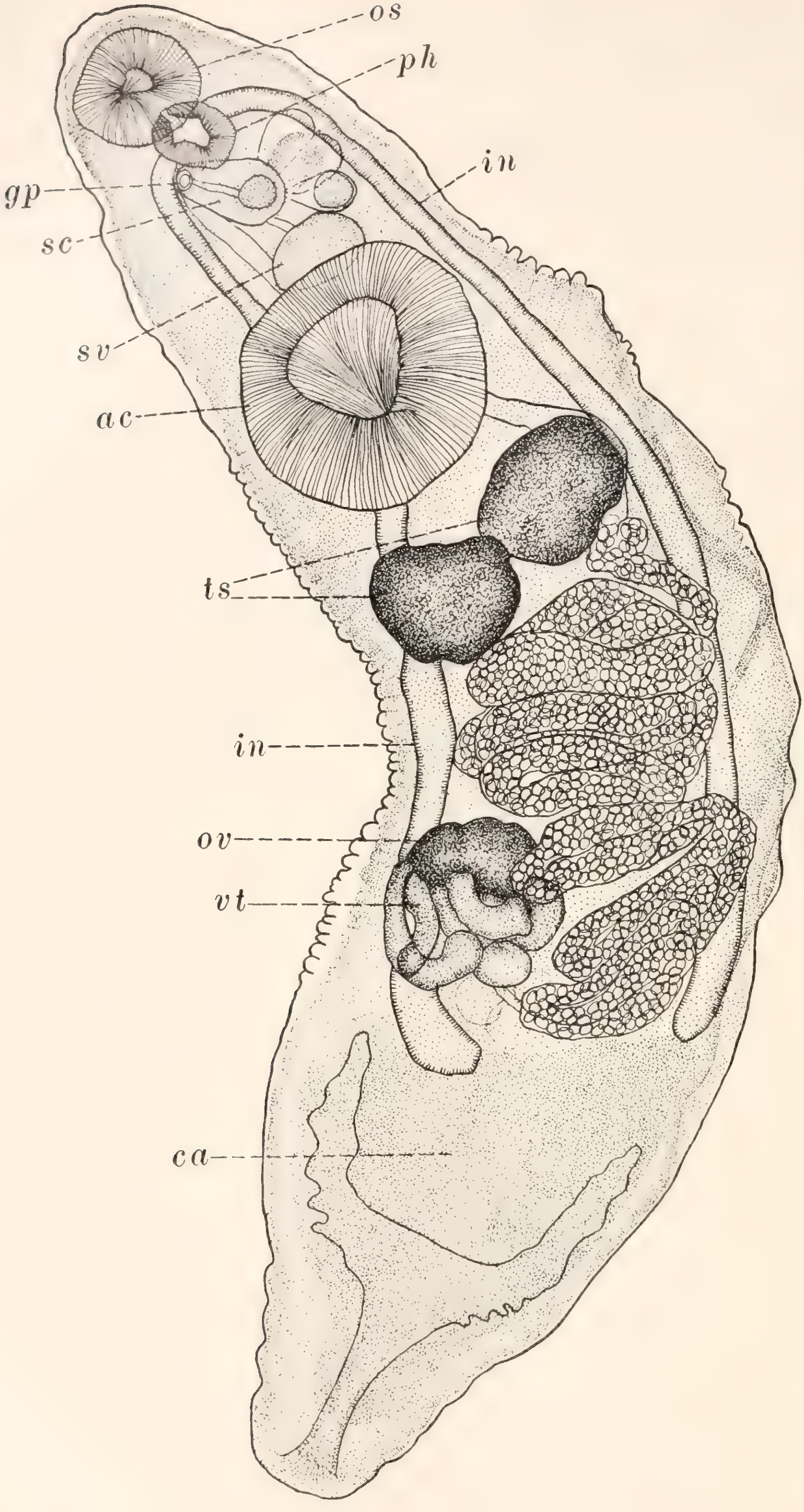
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FIG. 1. *S. branchialis,* ventral view, specimen 1.8 mm. long.

Throughout its length the duct is surrounded by the cells of the prostate gland. The copulatory organs and sinus sac agree in structure with those of *S. musculus* as described by Looss.

Female Reproductive System. The ovary is globular, somewhat ventral and to the right of the midline, usually some distance behind the testes but may be pushed forward on retraction of the " tail." It is slightly broader than long, about the same size as one of the testes. The vitellaria are lobular, posterior and adjacent to the ovary; arranged in two groups united by a short slender bridge, four lobes on the right and three on the left. The lobes are sometimes indistinct, spherical to oval, and may be three times as long as wide with thickened ends. The oviduct arises at the posterior end of the ovary and turns ventrad to join the oötype where a short vitelline duct is received. Mehlis' gland is present, the seminal receptacle and Laurer's canal absent. The initial portion of the uterus is filled with spermatozoa. The uterus is much coiled; it passes at first posteriad for a short distance and then forward to join the metraterm. The eggs are very numerous, 0.016–0.021 mm. in length by 0.01–0.013 mm. in width; average of 100 eggs, 0.019 by 0.012 mm.

Copulatory Organs. The genital pore is median, immediately behind the oral sucker. The sinus sac is pyriform, directed obliquely to the dorsal and anterior face of the acetabulum, between the intestinal rami. It is filled with loose, vacuolated parenchyma and contains the hermaphroditic duct, prostate vesicle, and terminal part of the metraterm. The arrangement of the fibers in the wall was described by Looss, who compared this structure with the closed cirrus sac of other digenetic trematodes. The hermaphroditic duct extends two-thirds to three-fourths of the length of the sinus sac, is almost straight, and usually contains eggs. It is undoubtedly formed by the fusion of the distal portions of the metraterm and ejaculatory duct. From the inner end of the hermaphroditic duct the metraterm extends caudad as a strong, straight tube. Dorsally, the short ejaculatory duct opens into the prostate vesicle, a spherical organ situated at the base of the sinus sac. The vesicle may contain spermatozoa, although usually it is either empty or partially filled with droplets of secretion from the prostate cells.

Excretory System. The excretory vesicle divides behind the acetabulum, and the crura unite dorsal to the pharynx. Further details of the system could not be observed.

Comparisons

As pointed out by Looss (1907*b*), the ability of the hemiurid trematodes to retract the posterior part of the body and telescope it into the major portion makes it exceedingly difficult to compare different species, and the criteria usually applied to other distomes can be utilized only with reservations. Very great changes in the shape and spatial relations of the internal organs are produced as the " caudal appendage " is protruded and retracted. These changes make it necessary to compare worms with similar degrees of contraction and frequently the limits of protrusion and retraction and corresponding internal changes can not be determined from fixed material alone. Only when an abundance of material is available can the observer distinguish with certainty between individual variations and specific distinctions. Comparison of specimens at different developmental stages is also helpful. Although specific determination is based primarily on morphological features, it is often facilitated by the biological characteristics of the specimens in question. Among parasitic species, the host-parasite relations have received much attention in recent years.

Sterrhurus branchialis differs in certain respects from each of the previously described species. Although other species have been found infrequently on the gills of their hosts, the chief seat of infestation is the stomach or adjacent portions of the digestive tract. In *S. branchialis,* conversely, the parasites occur primarily on the gills and were found in enormous numbers. Morphologically, *S. branchialis* is very similar to *S. musculus,* but the worms attain a larger size and the vitellaria are better developed. In Looss' figure of *S. musculus,* the vitelline glands are small, about twice as long as wide, the lobes are short, and their number was not stated.

S. branchialis is intermediate in size between *S. musculus* and *S. imocavus*. *S. imocavus* is slightly larger, more elongate, with larger suckers, smaller eggs, and less distinctly lobed vitellaria. Although the limits of body size overlap, *S. branchialis* differs from *S. grandiporus* in several respects. In *S. grandiporus* the suckers are relatively larger, the vitelline lobes are larger and longer, the " tail " is relatively smaller, and concomitant perhaps with the smaller " tail," more of the uterine coils are post-ovarian in position. *S. fusiformis* may be distinguished from

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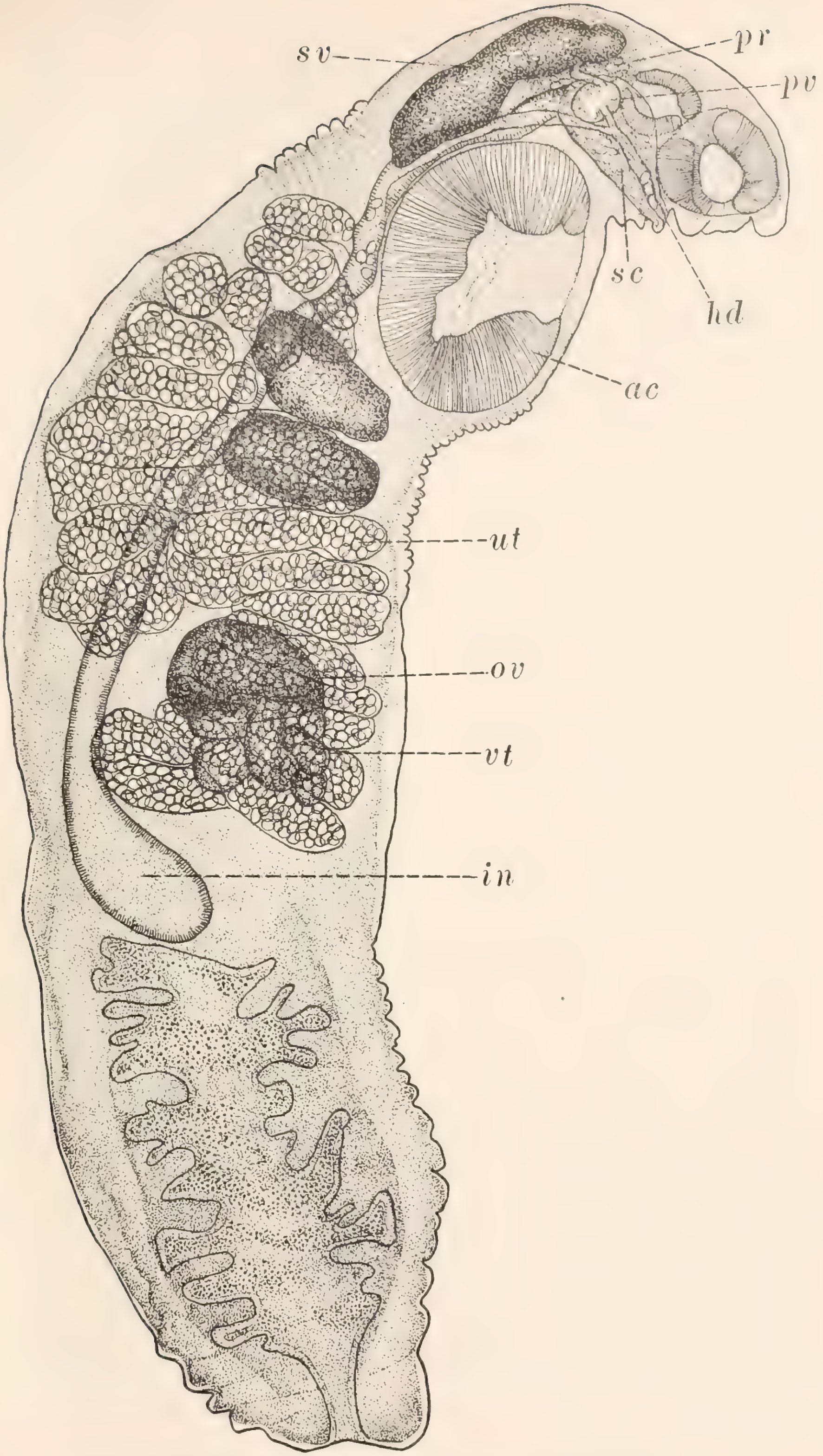
Abbreviations in Fig. 2

ac—acetabulum
ca—caudal appendage
gp—genital pore
hd—hermaphroditic duct
in—intestine
os—oral sucker
ov—ovary
ph—pharynx
pr—prostate gland
pv—prostate vesicle
sc—sinus sac
sv—seminal vesicle
ts—testis
ut—uterus
vt—vitellaria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in

FIG. 2. *S. branchialis,* from right side, specimen 2.15 mm. long.

S. branchialis since in the former species the worms are larger and the vitelline lobes are much longer and thinner. More than one species has undoubtedly been described as *S. monticellii* and the name should be restricted to the form originally described by Linton (1898) from *R. remora.* These specimens are almost twice the size of *S. branchialis* and show corresponding differences in size of internal organs. Otherwise the two species are very similar. Certain of the small specimens referred by Linton to *S. monticellii* were assigned by Manter (1934) to the species which he described as *S. floridensis.* This species, according to the author, differs from *S. musculus* only in size of eggs. *S. lævis,* as redescribed by Manter (1934), differs from *S. branchialis* in form of vitellaria, seminal vesicle, and sinus sac. *S. præclarus* differs from *S. branchialis* in form of copulatory organs and vitellaria, location of testes, and size of caudal appendage. *S. robustus* differs from *S. branchialis* in the absence of a sinus sac and in the location and form of the seminal vesicle. *S. branchialis* may be distinguished from *S. profundus* by differences in the position of the anterior limits of the " tail," and in the form of the copulatory organs and vitellaria.

Discussion

As noted by Odhner (1911), the Hemiuridæ are very similar and probably closely related to the Azygiidæ. In all of these trematodes the musculature is well developed and specimens assume varied forms on contraction. According to Ward (1917), " This (*Azygia*) is a powerfully muscular distome and may be greatly distorted in the process of preservation. Specimens taken from a single host at the same time and preserved in the same way often present marked external differences in size and form. The genera *Megadistomum* of Leidy and Stafford, *Mimodistomum* of Leidy, and *Hassalius* of Goldberger are instances of such extreme specimens that really belong to the single genus *Azygia.*" Indeed, the same condition was encountered in *S. branchialis.* Such great differences were found in the large number of worms studied that for a time it appeared that more than one species was represented. Different specimens, however, showed intermediate conditions and made it possible to assign all the material to a single species. This enormous variation among individuals of a single species makes it exceedingly difficult to distinguish between different species. Although they may be distinct and valid, we have attempted without success to formulate a satisfactory key to the several species of *Sterrhurus.*

In his diagnosis of the genus *Sterrhurus,* Looss (1907*b*) placed special emphasis on the form of the copulatory organs. He stated, " Der den Cirrusbeutel ersetzende Muskelsack umschliesst ausser dem Ductus hermaphroditus auch den Anfangsteil des Metraterms und den kurzen Ductus ejaculatorius, der seinerseits in den blasenartigen, das Hinterende des Cirrussacks einnehmenden Hohlraum übergeht. In diesen tritt von hinten die ausserhalb des Sacks gelegene Pars prostatica ein, wobei ihr innerer Belag muttermundartig in die Blase vorspringt."

The species *S. robustus, S. præclarus,* and *S. profundus* of Manter have copulatory organs which do not conform to the generic diagnosis and *S. profundus*, according to Manter, " differs from all others in the genus in the extreme development of the tail, the undivided seminal vesicle, the far forward position of the genital pore, the lack of prostate gland, the straight ascending limb of the uterus and the apparently ununited branches of the excretory vesicle. Its unlobed vitellaria are unusual as is also the extreme posterior extent of the uterus and ceca." The inclusion of these species in the genus *Sterrhurus* was made with the following explanation, " There arises a question as to whether the genus should be limited to species with sinus sac and terminal genital ducts as in *Sterrhurus musculus* Looss (the type species) and *Sterrhurus floridensis* (figs. 69, 70) or whether it should include also such forms as *Sterrhurus robustus, Sterrhurus præclarus,* and *Sterrhurus profundus.* During the early development of any genus, it is difficult to arrive at a logical generic limitation. As more species of *Sterrhurus* are named, the genus will undoubtedly be divided. At present it seems best to allow the genus to grow, based on the fundamental characters of smooth body provided with a tail appendage rather than to erect several genera of single species."

In our opinion the form of the copulatory organs is a feature of generic rank, and a group based only on a " smooth body provided with a tail appendage " is not a genus but deserves subfamily rank. Accordingly, since the species in question have copulatory organs which differ so fundamentally from those of *S. musculus,* we would not include these species in the genus *Sterrhurus.*

Since we have not examined material of other species of *Sterrhurus,* opinion concerning their validity can be based only on the published accounts in the literature. Many of the descriptions are brief and authors do not always agree on the dimensions given for a single species. A discussion of this subject may be found in the recent paper by Manter (1934). For example, the great variation in the dimensions recorded by Linton for the several forms described by him at various times as *Distomum monticellii* and the lack of complete descriptions make it difficult to characterize that species. Linton's (1898) measurements and figure differ considerably from those given in (1910) when he redescribed the parasite and placed it in the genus *Sterrhurus.* The specimens reported in 1898 are about four times as large as those described as *S. monticellii* in 1910. In his (1905) paper Linton described the parasite from a large number of hosts. His Fig. 154 was sketched from life with details added from fixed and stained specimens. The worm was taken from *Rachycentron canadus* at Beaufort, North Carolina. It was about the same size as the specimens described in 1898. The vitelline lobes as shown in this figure differ slightly from those of another specimen shown in Fig. 155 which had been taken from the sand pike, *Synodus fœtens.* It appears certain that more than one species must be represented in the material reported by Linton.

According to Manter (1934) the worms described as *D. monticellii* by Linton (1907) are probably specifically identical with *S. floridensis* Manter, 1934. But this species according to the author differs from *S. musculus* only in size of eggs. Looss gave the egg measurements of *S. musculus* as 0.019–0.021 mm. by 0.011–0.013 mm. The maximum egg length in *S. floridensis* is 0.017 and the greatest width about 0.01 mm. Most of the eggs measure from 0.013–0.015 mm. in length. The importance of egg measurements in determining specificity has been discussed by many authors. Cort (1915) noted the variation in egg size that may occur in a given species. In *S. branchialis* the egg measurements show variations that are close to the limits of all the described species. Size of eggs, in the absence of other morphological differences, is a very inadequate criterion by which to characterize a new species. This is true especially in cases where the life cycle is unknown. In the same species, e.g., *Fasciola hepatica* and *Clonorchis sinensis,* it has been demonstrated repeatedly that worms which have completed their development in different host species may show marked differences in size and in size of eggs. There is a further complicating possibility. It has been observed (see Stunkard, 1931) that trematode parasites of fishes may persist for a time in the digestive tract of piscivorous hosts and may become adapted to the new environment. Since the hosts of *Sterrhurus* feed in part at least on other fishes, such a method of disseminating the infestation can not be overlooked. In fact it is indicated by the fifty-five different species of fish which are listed by Manter as hosts of *S. floridensis.* An observation by Looss (1907*b*), " dass man bei jungen Sterrhurinen, die sich oft in Mengen encystiert im Peritoneum und anderen Organen verschiedener Fische finden," is of much significance in this connection. It indicates clearly that either different fishes normally serve as second intermediate hosts or that if ingested while young the parasites may encyst in these fishes awaiting a chance introduction into the proper host where they may complete their development. In the absence of knowledge concerning the life cycle and larval stages of these trematodes, specific identification must be largely tentative.

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Volume LXVII
Number 1

43522

THE
BIOLOGICAL BULLETIN

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AUGUST, 1934

Printed and Issued by
LANCASTER PRESS, Inc.
PRINCE & LEMON STS.
LANCASTER, PA.

THE BIOLOGICAL BULLETIN

THE BIOLOGICAL BULLETIN is issued six times a year. Single numbers, $1.75. Subscription per volume (3 numbers), $4.50.

Subscriptions and other matter should be addressed to the Biological Bulletin, Prince and Lemon Streets, Lancaster, Pa. Agent for Great Britain: Wheldon & Wesley, Limited, 2, 3 and 4 Arthur Street, New Oxford Street, London, W.C. 2.

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Volume LXVII Number 2

THE
BIOLOGICAL BULLETIN

PUBLISHED BY
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OCTOBER, 1934

Printed and Issued by
LANCASTER PRESS, Inc.
PRINCE & LEMON STS.
LANCASTER, PA.

THE BIOLOGICAL BULLETIN

THE BIOLOGICAL BULLETIN is issued six times a year. Single numbers, $1.75. Subscription per volume (3 numbers), $4.50.

Subscriptions and other matter should be addressed to the Biological Bulletin, Prince and Lemon Streets, Lancaster, Pa. Agent for Great Britain: Wheldon & Wesley, Limited, 2, 3 and 4 Arthur Street, New Oxford Street, London, W.C. 2.

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